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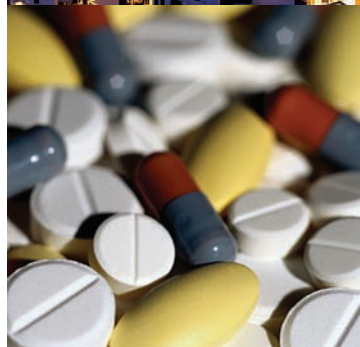
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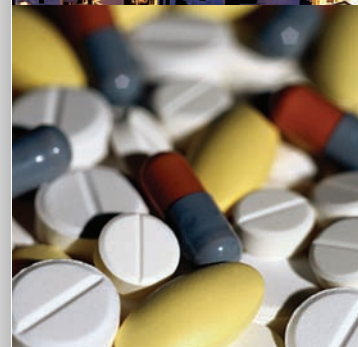
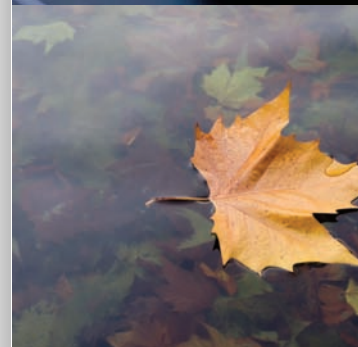
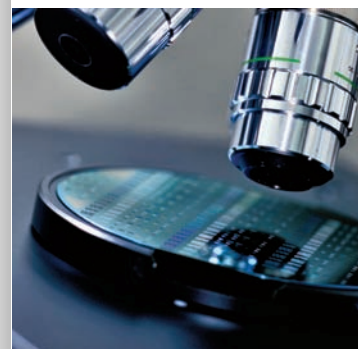
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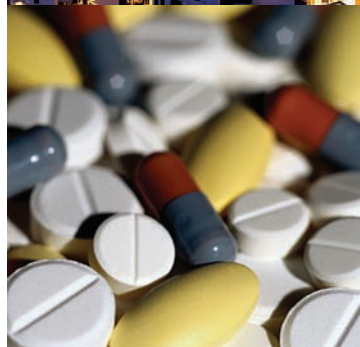
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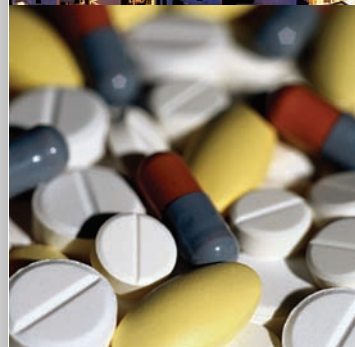
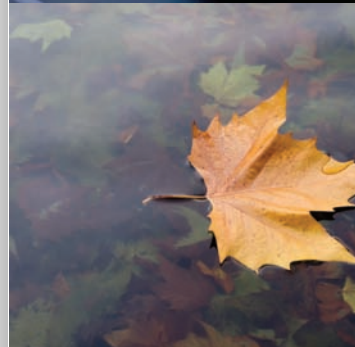
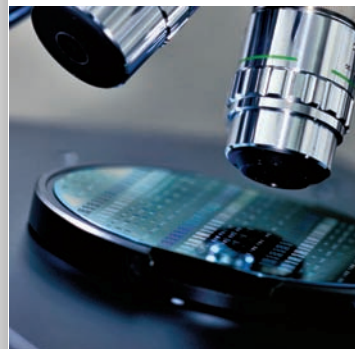
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Determination of Organochlorine Pesticides in Serum via QuEChERS and GC–MS

Xiaoyan Wang, UCT, LLC

Organochlorine (OC) pesticides are hydrocarbons with multiple chlorine substitutions primarily used as insecticides. OC pesticides do not break down easily as the chlorine-carbon bonds are very strong. They remain in the environment long after application and bio-accumulate in organisms long after exposure. They are toxic, and some are known or suspected human carcinogens, such as 4,4'-DDT, 4,4'-DDD, and the lindanes. Many OC pesticides are banned or have restricted use. These chemicals are frequently found in human body tissue, blood, fat, and breast milk. This application employs a modified QuEChERS extraction and a fast, push-through cartridge clean-up for the determination of OC pesticides in human serum.

Materials	
ECQUUS1115CT	15 mL centrifuge tube with 800 mg MgSO ₄ and 200 mg NaCl
ECPURMPSMC	Quick QuEChERS, medium push through cartridge with 110 mg MgSO ₄ and 180 mg PSA
GCLGN4MM-5	GC liner, 4 mm splitless gooseneck

Sample Preparation

- 1) Add 2 mL acetonitrile containing internal standard (IS) to a 15-mL centrifuge tube with 800 mg MgSO₄ and 200 mg NaCl (ECQUUS1115CT).
- 2) Add 1 mL serum sample and 1 mL reagent water into the 15-mL centrifuge tube. Shake for 1 min manually or use a Spex 2010 Geno-Grinder at 1000 strokes/min.
- 3) Centrifuge at 3000 g for 5 min.
- 4) Load 1.5 mL supernatant using a disposable syringe and attach a push through cartridge (ECPURMPSMC) to the Luer tip of the syringe.
- 5) Push the syringe plunger slowly, discard the first 3–5 drops (to remove any trapped airborne contaminants in the cartridge), then collect 0.5 mL extract into a 2-mL auto-sampler vial.

Instrumental

GC–MS: Agilent 6890N GC coupled to 5975C MSD

Injection: 1 µL splitless at 250 °C

Column: Restek Rxi®-5sil MS 30 m × 0.25 mm × 0.25 µm

Carrier gas: He at a constant flow of 1.2 mL/min

Oven temp program: 50 °C hold for 1 min, ramp at 10 °C/min to 310 °C and hold for 3 min

Detection: ESI/SIM mode, 25 ms dwell time

Results

Matrix matched calibration curves were constructed for analyte quantitation. The responses were linear over the analytical range from 20 to 2000 ng/mL with R² greater than 0.9970. The average

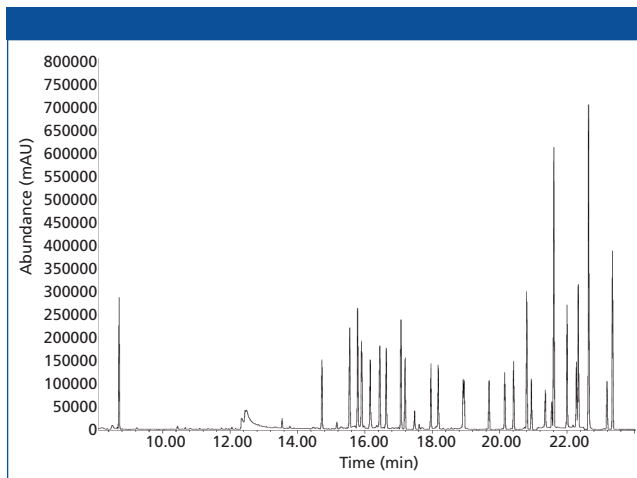


Figure1: Spiked serum sample.

recoveries (Rec%) and relative standard deviations (RSD%) of 5 replicated samples are listed in Table I.

Table I: Recovery and RSD% of spiked serum				
Compound	Spiked at 40 ng/mL (n = 5)		Spiked at 1000 ng/mL (n = 5)	
	Rec%	RSD%	Rec%	RSD%
alpha lindane	106.5	1.8	100.1	1.1
beta lindane	105.5	0.7	100.3	2.5
gamma lindane	110.2	1.9	101.2	1.3
delta lindane	101.4	1.5	101.0	1.0
Heptachlor	103.0	3.8	92.6	2.8
Aldrin	87.5	5.6	87.4	2.3
Heptachlor epoxide	98.7	2.2	99.6	2.5
trans Chlordane	90.1	3.2	92.7	2.6
cis Chlordane	90.8	3.4	92.5	1.4
Endosulfan I	96.9	3.1	94.4	1.3
4,4'-DDE	87.9	5.7	87.2	2.1
Dieldrin	101.5	0.7	95.1	4.2
Endrin	98.7	1.6	98.1	1.3
Endosulfan II	95.5	6.7	96.7	1.2
4,4'-DDD	77.8	2.6	94.6	1.1
Endosulfan sulfate	86.2	2.0	100.0	0.7
4,4'-DDT	61.6	4.7	87.0	5.1
Endrin ketone	91.7	2.2	99.5	1.4
Methoxychlor	69.8	2.3	96.0	0.7

Conclusion

This application provides a simple, fast, and efficient method for the determination of organochlorine pesticides in serum.



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TSKgel® Amide-80, 2 μm Columns for the Analysis of Glycans

Atis Chakrabarti, PhD, Tosoh Bioscience LLC

Most proteins, particularly soluble and membrane-bound proteins expressed in the endoplasmic reticulum, are glycosylated. The extent and result of glycosylation varies. For example, some proteins do not fold correctly unless they are glycosylated. The polysaccharide side chains (glycans) play critical roles in physiological and pathological reactions ranging from immunity to cell signaling, development, and death. The efficacy of biotherapeutic drugs is affected by glycosylation and therefore the profiling of the glycan pool released from a glycoprotein must be monitored to control product consistency.

Hydrophilic interaction chromatography (HILIC) with an amide-based stationary phase, used in conjunction with fluorescently labeled oligosaccharides, is a well-established, robust technique used by many laboratories to obtain high resolution separation of isolated glycans. TSKgel Amide-80 column chemistry is ideally suited for the separation of charged and neutral fractions of glycan pools in a single run. The application of a TSKgel Amide-80, 2 μm column for the analysis of glycans from monoclonal antibodies is demonstrated in this note, including a comparison to a 3 μm TSKgel Amide-80 column.

Materials and Methods

Figure 1

Columns: A: TSKgel Amide-80, 2 μm , 2.0 mm ID $\times$ 15 cm
B: TSKgel Amide-80, 3 μm , 2.0 mm ID $\times$ 15 cm
Mobile phase: A: 200 mmol/L acetic acid + triethylamine, pH 7.3
B: ACN
Gradient: 75% B (0–5 min)
75–50% B (5–80 min, linear)
Flow rate: 0.5 mL/min
Detection: fluorescence (λ_{ex} : 315 nm, λ_{em} : 380 nm)
Temperature: 40 °C
Injection vol.: 50 μL
Sample: pyridylaminated oligosaccharides released from mAb-1 (mouse)

Figure 2

Column: TSKgel Amide-80, 2 μm , 2.0 mm ID $\times$ 15 cm
Mobile phase: A: 200 mmol/L acetic acid + triethylamine, pH 7.3
B: ACN
Gradient: 75% B (0–5 min)
75–50% B (5–80 min, linear)
Flow rate: 0.5 mL/min
Detection: fluorescence (λ_{ex} : 315 nm, λ_{em} : 380 nm)
Temperature: 40 °C
Injection vol.: 50 μL

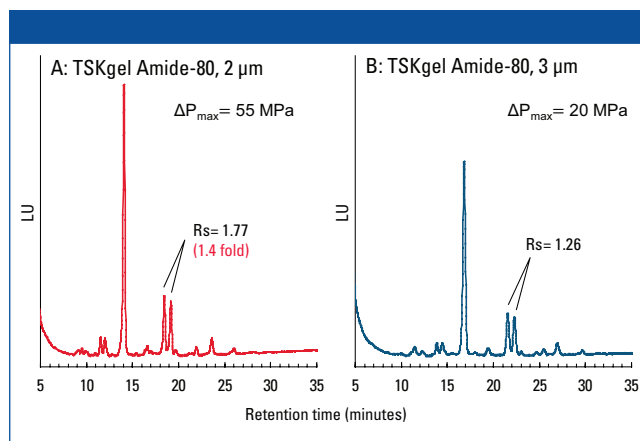


Figure 1: Separation of PA-glycans from mouse monoclonal antibody.

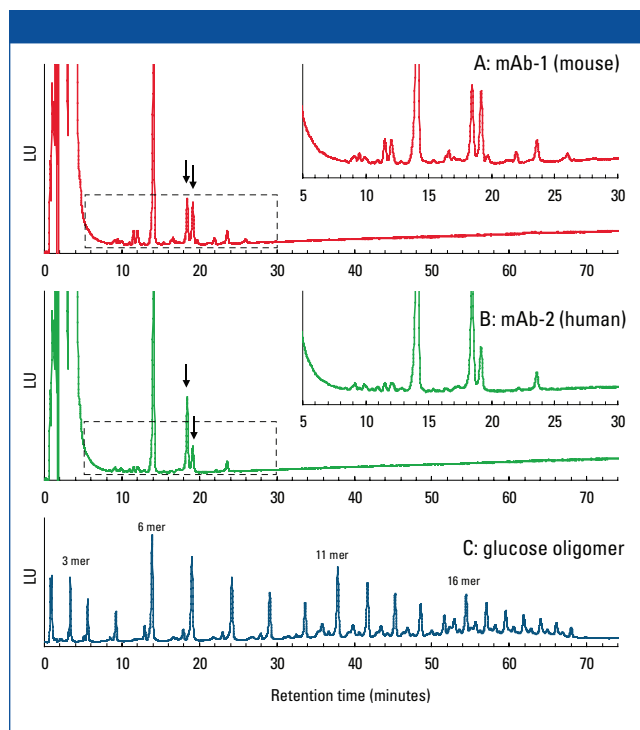


Figure 2: Comparison of glycan peak profile of monoclonal antibodies.

Samples: A: pyridylaminated oligosaccharides released from mAb-1 (mouse)
B: pyridylaminated oligosaccharides released from mAb-2 (human)
C: PA-glucose oligomer (3–22 mer) (TaKaRa Bio)

Results and Discussion

A comparison was done on the separation of pyridylaminated oligosaccharides released from mouse IgG on TSKgel Amide-80, 2.0 mm ID $\times$ 15 cm columns differing in particle size. As seen in Figure 1, the TSKgel Amide-80, 2 μ m showed a 1.4-fold higher resolution of PA-glycan peaks compared to the TSKgel Amide-80, 3 μ m column. With similar selectivity, method transfer from a 3 μ m to a 2 μ m column is easily accomplished. Note that the TSKgel Amide-80, 2 μ m column performed at a pressure of approximately 55 MPa during the gradient elution when run at the flow rate of 0.5 mL/min. A UHPLC system or a conventional HPLC system with a maximum pressure rating of greater than 40 MPa is required if the separation is run at this higher flow rate.

Pyridylation is a fluorescence-tagging method for oligosaccharides that enables measurement and structural analyses of glycans. Glycans do not contain fluorophores and thus must be labeled with fluorescent tags prior to analysis. 2-Aminobenzamide (2-AB) is one of the most common labels used.

Several peaks of glycans were separated from both mouse IgG and human IgG (Figure 2). These peaks were similar in elution time to 6-8 mer glucose. When comparing chromatograms A and B, different ratios of glycan attached to each mAb is implied.

Conclusions

A TSKgel Amide-80, 2 μ m column has similar selectivity as an Amide-80, 3 μ m column. The smaller particle size is ideally suited for the higher resolution separation of glycans. This 2 μ m column showed much higher resolution than a TSKgel Amide-80, 3 μ m column for the separation of pyridylaminated oligosaccharides released from mouse IgG and successfully separated several peaks of glycans from both mouse IgG and human IgG. Because of equivalent selectivity, a smooth method transfer from a 3 μ m TSKgel Amide-80 column to a 2 μ m column is an additional advantage.

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High-Speed SFC Enantiomeric Separation Using the Optimal Daicel SFC Chiral Columns

Chiral Technologies, Inc.

Supercritical fluid chromatography (SFC) is an evolutionary technology and a powerful tool for enantiomer separation when used in combination with chiral stationary phases (CSPs).

In parallel to the recent advancements in SFC instruments, there has been significant evolution of chiral columns in terms of CSP enantioselectivity, versatility, column stability, and efficiency. In order to provide optimal SFC chiral columns for ultra-fast analysis, the product line of chiral columns based on polysaccharide derivatives has recently been extended to new column dimensions.

Experimental and Discussion

As shown in the separation examples, Daicel chiral columns packed with 3- μm CSP particles and sized to 3.0-mm i.d. $\times$ 100 mm long can take full advantage of state-of-the-art SFC instrumentation to achieve fast and ultra-fast chiral separations without compromising the optimal resolution of enantiomers. These beneficial properties can be attributed to the high enantirecognition ability of the CSPs, van Deemter fast mass-transfer kinetics, column packing stability, and the selection of column diameter.

Chromatographic Conditions

Columns:	Daicel 3- μm CHIRALPAK® AD-3 and CHIRALPAK IC-3, 3-mm i.d. $\times$ 100 mm long
Mobile phase:	CO_2/MeOH 85:15 (by volume)
Additive:	(a) 1% DEA in MeOH; (b) None
Pressure:	150 bar
Flow rate:	3.6 mL/min
Temperature:	40 °C
Detection:	UV
SFC System:	AQUITY UPC2®

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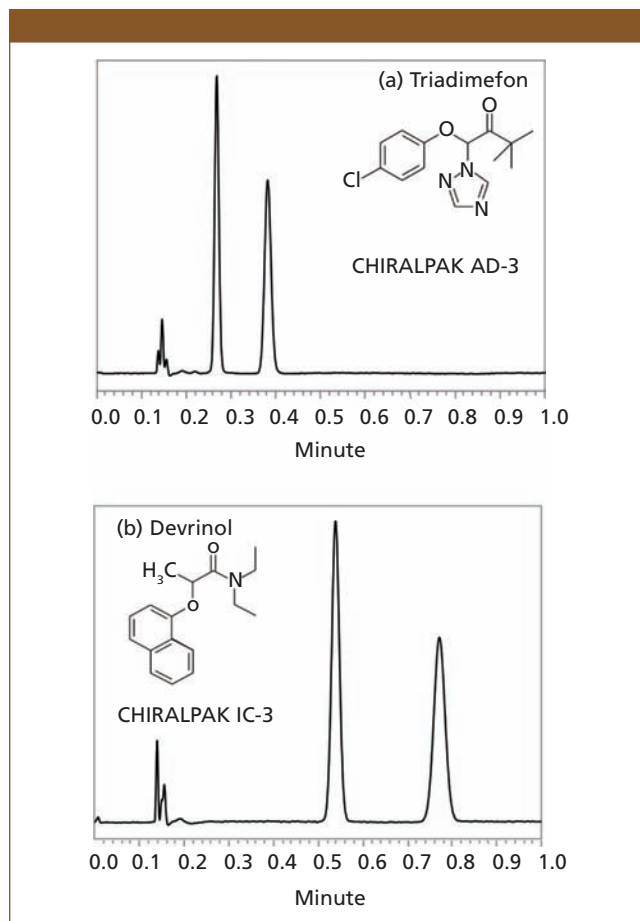


Figure1: Examples of fast chiral analysis by SFC.


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Chiral SFC Separation of Chemical Warfare Nerve Agents Using the Whelk-O®1 Chiral Stationary Phase

Shane A. Kasten et al.* and Angie Thayer†, *Physiology and Immunology Branch, Research Division, US Army Medical Research Institute of Chemical Defense, APG and †Regis Technologies, Inc.

Chemical warfare nerve agents (CWNAs) are extremely toxic organophosphorus compounds that contain a chiral phosphorus center. Because exposure of soldiers and civilians to CWNAs as a result of potential terrorist and other activities is a serious threat, the toxicity of these compounds has been studied extensively. The US Army Medical Research Institute of Chemical Defense recently demonstrated the effectiveness of the Whelk-O®1 Chiral Stationary Phases (CSPs) for the separation of a class of extremely toxic chiral organophosphorus (OP) compounds called G-type CWNAs (*Chirality*, **26**[12], 817–824 [2014]). Racemic mixtures of tabun (GA), sarin (GB), soman (GD), and cyclosarin (GF) were screened against a variety of CSPs for baseline resolution. Whelk-O®1 (S,S) was shown to achieve baseline resolution for GA, GB, and GF when two columns were run in tandem, resulting in the P(+) isomer eluting first. This approach was also applied to the separation of the four diastereomers of GD and resulted in baseline separation of the two P(+) epimers.

Experimental Conditions

The enantiomers of GF were screened against a variety of CSPs using 0% or 1% isopropanol cosolvent to identify a column capable of partial resolution ($R_s \sim 1.0$) at minimum. Further optimization focused on establishing full baseline resolution ($R_s \geq 1.6$) of the stereoisomers of GF followed by methods for GA, GB, and GD using the Whelk-O®1 (S,S) column. A variety of cosolvent alcohols, using 0% or 1%, were used to evaluate separation efficiency of racemic GF on the Whelk-O®1 (S,S) column. Full baseline resolution ($R_s = 1.7$) was only achieved with 0% cosolvent when a single Whelk-O®1 (S,S) column was used.

A second column was installed in series to increase the column bed length and improve the separation of isomers. The baseline resolution for GF enantiomers was increased to $R_s = 2.2$ with 0% cosolvent. Full baseline separation ($R_s = 1.7$) was only achieved with IPA cosolvent on the tandem Whelk-O®1 (S,S) columns. None of the compounds could be fully resolved on a single column, even with 0% cosolvent. Upon evaluation with the second column in tandem, resolution was achieved for GA, GB, and the first two isomers of GD (Figure 1).

Conclusion

The Whelk-O®1 was identified as a suitable CSP for the enantiomer separation of CWNAs. Further method development demonstrated that the Regis Whelk-O®1 (S,S) columns were capable of analytical chiral separation of GA, GB, GD, and GF via SFC. The Regis Whelk-O®1 (S,S) column achieved baseline separation of the GA, GB, and GF enantiomers and the P(+) enantiomers of GD. In addition, preliminary method development on the semipreparative scale has already proven successful, providing a means for isolating pure enantiomers on the milligram scale.

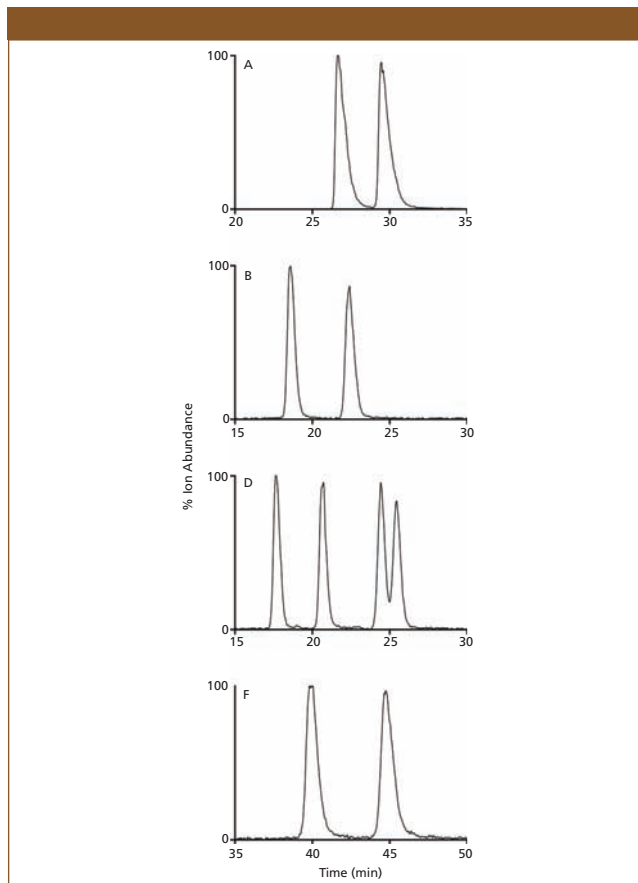


Figure 1: Separation efficiency profiles for GA (A), GB (B), and GD (C). Stereoisomers were separated using 2x Whelk-O®1 (S,S) columns with 0% cosolvent.

Disclaimer

The views expressed in this presentation are those of the authors and do not reflect the official policy of the Department of Army, Department of Defense, or the U.S. Government.



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How to Comply with Method 325 for Refinery Fenceline Monitoring

Caroline Widdowson and David Barden, Markes International

Complying with US EPA Method 325 for monitoring benzene and other volatile organic compounds (VOCs) at refinery perimeters might at first sight seem daunting. However, establishing a protocol in accordance with Method 325 need not be complex, and this article outlines a fully compliant, stepwise approach for sampler deployment, sample analysis, and tube cleaning.

US EPA Method 325 (1) has been developed to enable refineries to comply with the updated US federal regulation CFR 40, and states that monitoring of benzene around the boundary of refineries will be compulsory from mid-2015. This will require two-week passive (diffusive) sampling onto sorbent tubes, followed by GC analysis.

This article will break down the seemingly complex requirements of Method 325 into a straightforward, four-step process (Figure 1), and highlight the key issues that should be considered in order to achieve full compliance.

A Stepwise Process for Sampling and Analysis

Step 1: Field Sample Deployment

Method 325 states that up to 24 monitoring locations should be distributed around the perimeter (fenceline) of the refinery, in a pattern that depends upon the size and shape of the site. Additional monitoring stations may also be required in certain circumstances.

The shelters that house the sampling tubes should be mounted on 1.5–3 m poles located around the perimeter of the facility. Such shelters should obviously be weather-proof, constructed of non-emitting materials such as stainless steel, and big enough to hold samples, replicates, and blanks. In accordance with standard practice in diffusive monitoring, such shelters should be positioned away from trees, buildings, or other obstructions that may result in local deviations in ambient air concentration.

The samplers themselves are fitted with a diffusive cap at the sampling end, which points down within the shelter. Doing this not only prevents particles from entering, but also eliminates air turbulence inside the tube, ensuring that the rate of adsorption of air pollutants (the “uptake rate” [2]) stays constant.

Step 2: Passive Sampling

The sampling protocol stipulated in Method 325 uses 3½-inch by ¼-inch stainless steel tubes packed with a single bed of sorbent. Medium-strength graphitized carbon blacks are the most appropriate sorbents for refinery monitoring, because they are able to trap benzene as well as a range of other relevant compounds, including butadiene, toluene, ethylbenzene, and the xylenes.

Once in the field, the clean, conditioned, and capped sorbent tubes are allowed to equilibrate at ambient temperature before re-



Figure 1: The stepwise sampling and analytical process enabling compliance with Method 325.

moval from the storage container, to minimize risk of condensation. The storage cap at the sampling end of the tube is then removed and replaced with a diffusion cap. Field blank tubes must be deployed at the same time as the sampling tubes, and on these tubes the long-term storage caps stay fitted to both ends.

Sampling end time and date information is usually logged at this stage, either manually or electronically. However, the large number of samplers required by Method 325 makes manual record-keeping burdensome, so to minimize costs and reduce the risk of human error, tube-tagging systems are recommended. Robust radio-frequency identification tags are attached to each tube and used to enter tube and sampling information. Such technology allows the development of a robust “chain of custody” from field to lab and ensures a verifiable method for audit trails.

If more than one sampler is being used at a given monitoring station, they should all be deployed in quick succession, so they effectively have the same start time. Sampling is carried out for a minimum of 14 days, during which time gaseous VOCs migrate into the air gap inside the tube, and adsorb onto the sorbent. At the end of the sampling period, the diffusion caps are removed from the sample tubes and replaced with the long-term storage caps, ready for transport to the analytical laboratory.

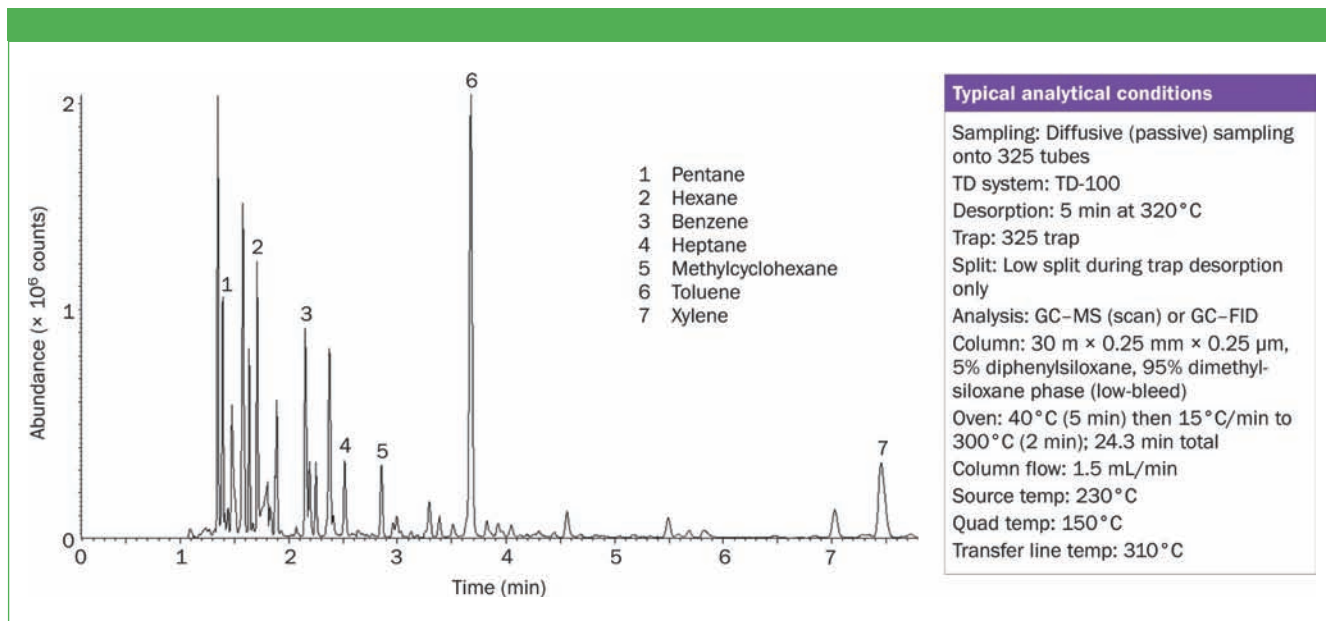


Figure 2: The results of two-week passive sampling of contaminated air around a refinery perimeter, with analysis by TD-GC-MS, showing the detection of a number of hazardous VOCs including benzene, toluene, and xylene.

Step 3: Sample and Data Analysis

When the sampled tubes reach the laboratory, they are analyzed using thermal desorption (TD)-GC, typically with MS detection. The large number of samples required by Method 325 means that automated analysis is essential, but instruments are available that can run 100 sample tubes in succession, reducing running costs and allowing unattended operation over entire weekends.

While the primary objective of Method 325 is to quantify the level of benzene, the sampling and analytical techniques described above also allow the simultaneous analysis of a wide range of compounds using the same workflow, without additional effort or cost (Figure 2).

Step 4: Sample Tube Cleaning

Method 325 states that sorbent tubes must be cleaned ("conditioned"), and demonstrate ≤0.2 ppbv of any contaminants or interferences before use. This can be done on the analytical instrument, but using a dedicated multi-tube conditioner frees up instrument time to run samples rather than condition tubes.

Conclusions

This article has shown that the technologies to comply with US EPA Method 325 are readily available and well-validated. The straightforward four-step protocol we have outlined here will enable refineries to achieve full compliance with the method, with all equipment described being available directly from Markes International.

Markes International has extensive experience of consulting on standard/regulatory methodology in the USA and worldwide, which places us in a very good position to advise refineries and contract laboratories on the best way to comply with Method 325.

References

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- (2) A large number of uptake rates have been published – see Markes International's Application Note 001, <http://www.markes.com/Resources/Application-notes/Technical-support.aspx>.

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LC-MS-MS Measurement of EPA Method 1694 Pharmaceuticals and Personal Care Products in Water

Jeremy Post, Shimadzu Scientific Instruments

This method increases sensitivity and reduces data acquisition time for a subset of pharmaceuticals and personal care products from the original EPA Method 1694 specifications.

Both pharmaceuticals and personal care products have increasingly passed into our nation's waters since their first use. Though present in low concentrations, some of the thousands of these chemical species are suspected of impacting both plant and animal health. To effectively study the impacts of these chemicals on the environment, a sensitive and selective analytical method utilizing a LCMS-8050 UHPLC-triple quadrupole mass spectrometer demonstrated here can monitor these species at low levels. In addition to improved sensitivity, the ultrafast instrument operating speed utilizes methods that are 7–8 times shorter than those in the original work.

Experimental Conditions

Method 1694 measures analytes from many disparate compound classes and therefore subdivides each sample into groups utilizing four unique extractions. This work demonstrates analytes from groups 1 (acidic extraction, positive polarity) and 3 (acidic extraction, negative polarity). The mobile phases best suited for their measurement differ, requiring two methods. An Eclipse Plus C18 1.8 μ 2.1 $\times$ 150 mm column was used in both methods, flowing at 0.3 mL/min, 40 °C. Source temperatures including a heated ESI

probe, heat block, and desolvation line along with heating, nebulizer, and drying gas flows and interface voltages were optimized. The autosampler tray was kept at 4 °C.

Results

Using mobile phases dedicated to both low and high pH in two methods improved the sensitivity for each analyte significantly (data not shown). Table I provides a comparison of method parameters and minimum levels of quantitation (ML) (1) together with the range of concentrations included in the calibration curve for each analyte with its coefficient of determination.

Conclusions

Utilizing Method 1694 as a foundation, pharmaceuticals and personal care products can be sensitively quantitated on the Shimadzu LCMS-8050

Reference

- (1) http://water.epa.gov/scitech/methods/cwa/bioindicators/upload/2008_01_03_methods_method_1694.pdf



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Table I: Comparison between LCMS-8050 and Method 1694 parameters and results

Compound	Molecular Weight	1694 Group	Polarity	Transitions	1694 Injection Volume (μ L)	8050 Injection Volume (μ L)	1694 ML ¹ on column (fg)	8050 ML ¹ on column (fg)	% improvement 8050 ML vs. 1694 ML	8050 Calibration Curve Range (ng/mL)	r^2
Acetaminophen	151.2	1	+	2	15	1	3000	2500	17	2.5-50	0.9999
Caffeine	194.2	1	+	2	15	1	750	250	67	0.25-50	0.9998
Carbamazepine	236.3	1	+	2	15	1	75	5	93	0.005-50	0.9994
Fluoxetine	345.8	1	+	2	15	1	150	100	33	0.1-50	0.9999
Sulfachloropyridazine	284.7	1	+	2	15	1	75	50	33	0.05-50	0.9998
Sulfadiazine	250.3	1	+	2	15	1	75	25	67	0.025-50	0.9999
Sulfamethazine	278.3	1	+	3	15	1	30	25	17	0.025-50	0.9999
Trimethoprim	290.3	1	+	3	15	1	75	25	67	0.025-50	0.9999
Gemfibrozil	250.3	3	-	2	15	1	75	50	33	0.05-50	0.9936
Ibuprofen	206.3	3	-	1	15	1	750	100	87	0.1-50	0.9925
Triclosan	289.5	3	-	1	15	1	3000	2500	17	1.0-50	0.9993

Fingerprinting Analysis of Different Types of Beer

Sonja Krieger,

Agilent Technologies Inc., Waldbronn, Germany

This application note demonstrates the comprehensive 2D-LC analysis of different types of beer. Nontargeted, multisample analysis (fingerprinting analysis) enabled a classification of the different types of beer analyzed.

Beer is an alcoholic beverage produced by saccharification of starch and fermentation of the resulting sugar. The basic ingredients of beer are water, malted barley or wheat, and for most beers hops (1). The typical beer bitterness is achieved by adding hops (*Humulus lupulus* L.) as cones, pellets, or extracts during wort boiling and results from iso- α -acids (isohumulones) and polyphenolic compounds (2,3). Because of the highly complex composition of beer, comprehensive 2D-LC is ideally suited for a comprehensive analysis of beer.

Experimental Conditions

Comprehensive 2D-LC analysis was achieved with the Agilent 1290 Infinity 2D-LC solution. In the first dimension an Agilent ZORBAX Narrow-Bore RR Extend-C18 column (2.1 $\times$ 100 mm, 3.5 μ m) was used with a gradient of 5 mM ammonium acetate in water, adjusted to pH 9.95 with ammonia, and acetonitrile/ethanol (60/40, v/v) at a flow rate of 0.075 mL/min. The second-dimension separation used an Agilent Poroshell HPH-C18 column (4.6 $\times$ 50 mm, 2.7 μ m) with shifted gradients of water and acetonitrile, each with 0.25% formic acid, at a flow rate of 4.0 mL/min. Modulation was realized using the Agilent 2-position/4-port duo-valve, equipped with two 60- μ L loops. A modulation time of 21 s was deployed. Diode array detection at 270 nm as well as mass spectrometric detection in negative ionization mode was performed. Beer samples were degassed and filtered before injection into the HPLC system.

Results

A comprehensive 2D-LC method was developed for the fingerprinting analysis of different types of beer. Panel A in Figure 1 exemplarily shows the chromatogram of the analysis of one German weizen beer with UV detection at 270 nm. It can be seen that a good coverage of the two-dimensional separation space was achieved by using C18 columns with alkaline and acidic pH values in the first- and second-dimension separations, respectively.

For comparison and classification of the different beer samples analyzed, a nontargeted, multisample analysis comparing every constituent in every sample was used. For this purpose, a cross-sample feature matching with generation of a composite chromatogram was performed. The composite chromatogram contains all peaks from all chromatograms and is used to define peak-region features (feature areas). For each chromatogram, the percent response for each of the defined feature areas is calculated. Classification of the analyzed beer

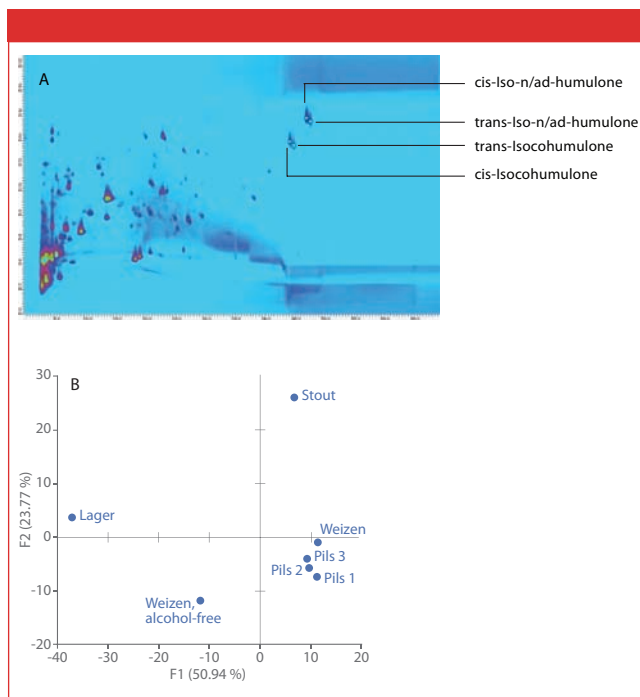


Figure 1: (A) Comprehensive 2D-LC analysis of German weizen beer with UV detection at 270 nm; (B) Principal component analysis of seven different beer samples.

samples was performed by principal component analysis (PCA) using the defined feature areas with their respective percent responses for every sample. Using PCA, the beer samples could be classified according to their type, as shown in panel B of Figure 1.

Conclusions

The comprehensive 2D-LC analysis of different types of beer using C18 columns at alkaline and acidic pH values in the first and second dimension, respectively, provides good orthogonality. By nontargeted, multisample analysis followed by principal component analysis, it was possible to classify the different types of beer analyzed.

Learn More

Download the application note at www.agilent.com/chem/5991-5521EN

References

- (1) A. S. Araujo, et al., *Analyst* **130**, 884–889 (2005).
- (2) D. Intelmann, et al., *J. Agric. Food Chem.* **57**, 1172–1182 (2009).
- (3) L. Ceslova, et al., *J. Chromatogr. A* **1216**, 7249–7257 (2009).



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Analysis of Nitrosamines in Malt

Andrew James, Ellutia Inc.

In brewing, barley undergoes a malting process, the grain is first drenched to make it germinate and then dried in kilns to capture the sugar and flavor compounds for the brewing process. The heating in the kilns can cause the formation of carcinogenic nitrosamine compounds in the grain, these can then potentially be passed on to the final brewed product. It is therefore essential that any malt used in brewing is checked for nitrosamine levels. The most common of the nitrosamines formed is N-nitrosodimethylamine (NDMA).

While GC-MS can be used to perform this analysis, the systems are costly to purchase and often need to be used in single ion monitoring mode to achieve the required sensitivity for NDMA. This means that other nitrosamines formed could be potentially missed. The Ellutia 800 Series TEA is an alternative detector that is able to achieve the required sensitivity for nitrosamine compounds, while also being easier to operate and maintain than a GC-MS system.

Experimental Instrument Conditions

The Ellutia 200 Series GC was interfaced with an Ellutia 810 TEA detector. The column used for the analysis was a 30 m DB-waxETR 0.25 i.d. $\times$ 0.25 μ m. The method settings were as per (Table I).

Sample Preparation

A combination of 75 g of malted barley with 250 mL of water and 250 μ L of internal standard (N-nitrosodipropylamine (NDPA) 1000 ng/mL) is homogenized in a blender. This is then filtered, and a liquid-liquid extraction into dichloromethane is performed. This extract is then evaporated down to 1 mL using a rotary evaporator. The sample is then ready to be analyzed by direct injection to the GC.

A number of standard solutions of known quantities of NDMA ranging from 0.5 to 10 ppb are prepared. These are then added to 75 g of NDMA-free malt and 250 mL of water and prepared using the same blending and extraction method. These are then used to prepare a standard calibration curve.

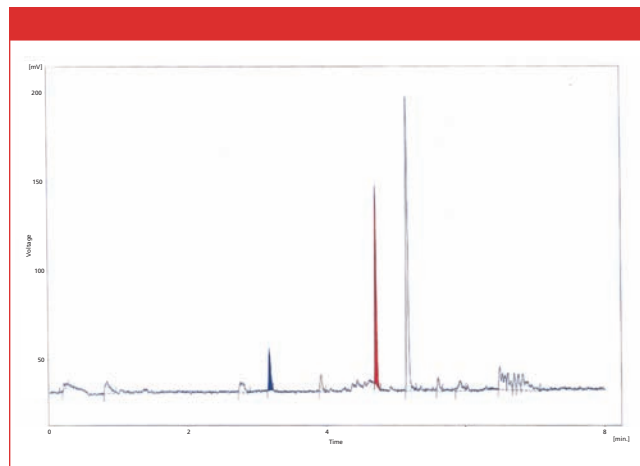


Figure1: NDMA in malt.

Results

The system was able to analyze the compounds of interest down to the required low ppb levels. Figure 1 shows a sample of malt showing 2.75 ppb of NDMA.

Conclusions

The Ellutia 200 Series GC and 800 Series TEA can provide a solution for nitrosamine analysis at the low levels required as an alternative to more expensive GC-MS systems. The TEA also allows other nitrosamine species to be monitored during a single run.

Table I: GC method

Injector mode	Splitless
Injector temperature	200 °C
Column temperature program	90 °C hold for 0 min 25 °C/min to 210 °C Hold at 210 °C for 3 min
TEA pyrolyzer temperature	500 °C



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Arsenic Speciation on Hamilton PRP-X100

Hamilton Company

Organic arsenic compounds added to feed stocks of chicken and poultry pose serious environmental and ecological threats. National and worldwide health organizations, such as the United States Environmental Protection Agency and the US Food and Drug Administration, have recently implemented more stringent concentration limits for arsenic species in drinking water and foodstuffs.

Analysis of arsenic species is made challenging due to diverse sample matrices. To circumvent these problems, an HPLC ICP-MS method has been developed. Resolution of arsenic compounds is achieved by ion exchange chromatography on a Hamilton PRP-X100 column, a 55% cross-linked polystyrene-divinylbenzene copolymer functionalized with quaternary ammonium anion-exchanger group. Detection is accomplished by inductively coupled plasma-mass spectrometry.

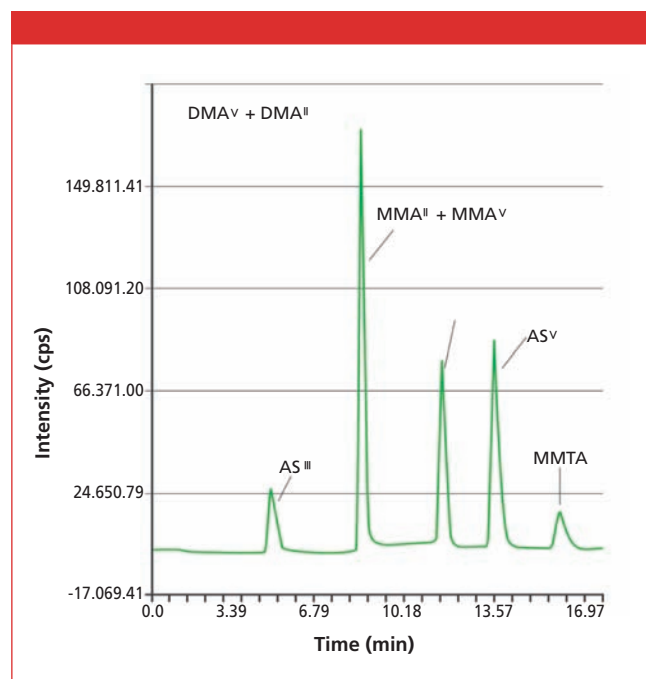


Figure 1: Borrowed and adapted (with permission) from P. Alava et al., *Biomed. Chromatogr.* **26**, 524–533 (2012).

Table 1: Chromatographic conditions

Column:	Hamilton PRP-X100, 5 μ m, 4.6 $\times$ 250 mm
Part Number:	79181
Flow Rate:	1.0 mL/min
Mobile Phase:	2 mM (NH ₄) ₂ CO ₃ for 0–3 min 40 mM (NH ₄) ₂ CO ₃ for 3–14 min 2 mM (NH ₄) ₂ CO ₃ for 13–17 min
Injection Volume:	50 μ L, 100 μ g/L of each standard
Detection:	ICP-MS

Ordering Information

Part Number	Product Name	Particle Size	Dimensions	Material
79852	PRP-X100	5 μ m	2.1 $\times$ 150 mm	PEEK
79190	PRP-X100	5 μ m	2.1 $\times$ 250 mm	Stainless Steel
79810	PRP-X100	5 μ m	4.1 $\times$ 50 mm	Stainless Steel
79538	PRP-X100	5 μ m	4.1 $\times$ 100 mm	Stainless Steel
79812	PRP-X100	5 μ m	4.1 $\times$ 150 mm	Stainless Steel
79174	PRP-X100	5 μ m	4.6 $\times$ 150 mm	PEEK
79181	PRP-X100	5 μ m	4.6 $\times$ 250 mm	PEEK
79421	PRP-X100	10 μ m	2.1 $\times$ 150 mm	Stainless Steel
79346	PRP-X100	10 μ m	2.1 $\times$ 250 mm	Stainless Steel
79365	PRP-X100	10 μ m	4.1 $\times$ 50 mm	Stainless Steel
79439	PRP-X100	10 μ m	4.1 $\times$ 100 mm	Stainless Steel
79434	PRP-X100	10 μ m	4.1 $\times$ 150 mm	Stainless Steel
79433	PRP-X100	10 μ m	4.1 $\times$ 250 mm	Stainless Steel
79354	PRP-X100	10 μ m	4.6 $\times$ 150 mm	PEEK
79455	PRP-X100	10 μ m	4.6 $\times$ 250 mm	PEEK
79353	PRP-X100	12-20 μ m	21.2 $\times$ 250 mm	Stainless Steel

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Automated Solid Phase Extraction for Efficient Extraction of Fluoroquinolone Antibiotics from Fish

Toni Hofhine*, Curtis Hedman†, David Rogers†, Robert Bucu‡, Richard Koeritz‡, Zachary Lilla‡, Michael McGinley§, Rob Freeman||, Ty Kahler||, and Rick Lake|| *Horizon Technology, Inc., †Wisconsin State Laboratory of Hygiene, ‡Shimadzu Corporation, §Phenomenex, and ||Restek

The global demand for fish as a natural source of fresh animal protein, essential fats, minerals, and vitamins continues to rise with the human population. It is estimated that natural fish resources will not be sustainable, creating the need to increase the available supply of fish through aquaculture. The mechanisms for keeping fish healthy and free of disease is regulated within each country, often prompting the use of antibiotics in the water; however, the misuse of large antibiotic doses has increased attention on the impact to human health and thus the testing of aquaculture according to country-specific food safety regulations.

Fluoroquinolones are a commonly used fluorine subclass group of quinolones that are effective against gram negative bacteria. Popular for their use in aquaculture, fluoroquinolones consumed in high levels have contributed to the development of antibiotic resistance in the human population. Concerns prompted the FDA to ban the use of fluoroquinolones in food producing animals in 1997. To regulate the use and level of fluoroquinolone antibiotics in fish from imports or aquaculture within the USA, the FDA routinely inspects domestic and imported aquaculture operations and recommends a standard method for the routine testing of four frequently used fluoroquinolone antibiotics in fish.

White bass filets represented low-fat fish and trout represented high-fat fish samples. Samples were extracted using the SmartPrep® Cartridge extractor (Horizon Technology) used with Strata-XL™-C and Strata-X-C cartridges (Phenomenex, Inc.). The final extract was analyzed with a Nexera XR HPLC (Shimadzu) using a biphenyl column (Restek).

Results

Results after method development show excellent recoveries as can be seen in Table I (1).

The larger pore and particle size of the Strata-XL-C SPE cartridges in combination with the SmartPrep Extractor System efficiently automated the combined LLE sample extracts to achieve consistent %RSD values across both high and low-fat fish matrices. Using new column technology with the UHPLC system improved peak resolution for increased sensitivity at the specified 20-ppb FDA monitored level. Elution of all four fluoroquinolone peaks occurred in 7 min compared to 20 min using the original column technology. The optimized method passed all FDA criteria for % recovery, % RSD, and linearity within less chromatographic

time and using current automation and consumable technology. Improving on workflow by implementing automation for the SPE process with the SmartPrep Extractor enabled objective sample handling across all samples by reducing scientist bias.

Reference

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Table I: Recoveries of four fluoroquinolones at 20 ppb using Strata-XL-C SPE cartridges

n = 3	Ciprofloxacin		Enrofloxacin		Sarafloxacin		Difloxacin	
Fish matrix	Low fat	High fat	Low fat	High fat	Low fat	High fat	Low fat	High fat
% Recovery	92.5	87.5	121.3	129.9	87.1	87.2	111.9	120.6
% RSD	0.88	1.90	0.42	3.94	2.45	2.03	2.25	2.99

Analysis of Sugar Alcohols by Semi-Micro HPLC

Yasuyo Sato, Keijin Iwaya, and DJ Tognarelli, JASCO

Semi-micro HPLC analysis offers a significant advantage compared with conventional separations in reducing separation time and cutting cost by reducing the consumption of mobile phase. However, HPLC systems designed for conventional separations often do not provide good enough peak shape or separation due to peak diffusion in the non-optimized flow path. When using a semi-micro column an optimized RHPLC system is recommended. This analysis report compares the measurement of sugar alcohols using a conventional refractive index detector with a detector optimized for semi-micro HPLC.

Experimental

Equipment

Pump: PU-4185
 Pump option: Degasser unit
 Autosampler: AS-4150
 Column oven: CO-4060
 Detector: RI-4035

Conditions

Column: Inertsil Amide (2.1 mm I.D. × 250 mm/L, 3 μm)
 Eluent: Water/acetonitrile (30/70)
 Flow rate: 0.2 mL/min
 Column temp.: 30 °C
 Injection volume: 1 μL
 Standard Sample: Sugar alcohols mixture (5 mg/mL each)

Table 1: System configuration

P/N	Description
7003-J014A	PU-4185 RHPLC semi-micro pump (base unit)
7006-H003A	DG-4000-04 4-channel degasser unit, for analytical
7064-J002A	AS-4150 RHPLC autosampler
7021-J002A	CO-4060 column oven
7031-J002A	RI-4035 refractive index detector, for semi-micro LC
7058-J011A	BS-4000-1 bottle stand
6688-H564A	LC-net CG cable (3x)
7059-J012A	ChromNAV Ver.2 chromatography data system
7001-H403A	RHPLC/UHPLC start up kit for LC-4000
7001-H405A	Maintenance tool kit
	Inertsil Amide (2.1 mm I.D. × 250 mm/L, 3 μm)

Results

Figure 1 shows the chromatograms of sugar alcohols measured using a refractive index detector for conventional analysis (RI-4030) or for semi-micro analysis (RI-4035). Using the RI-4035 for semi-micro analysis can obviously provide improved peak shape and resolution.

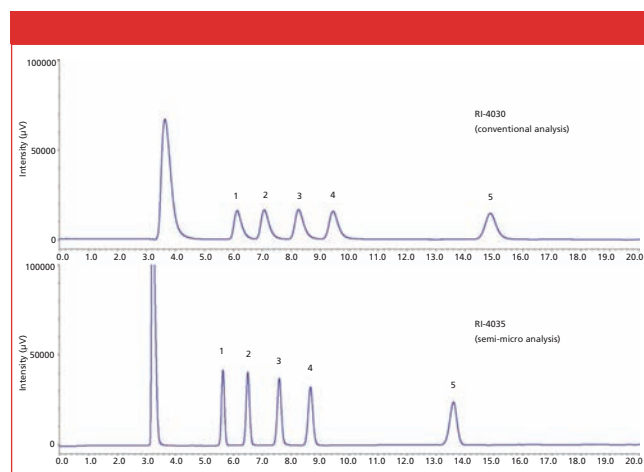


Figure 1: Chromatogram of sugar alcohol samples 1: glycerine, 2: erythritol, 3: xylitol, 4: solbitol, 5: inositol.



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A New HILIC Column for Saccharide Analysis

Melissa Turcotte* and Naoya Nakajima†,

*Showa Denko America, Inc., and †Showa Denko K.K.

Due to the highly polar nature of saccharides, the analysis of sugars is typically achieved using hydrophilic interaction chromatography (HILIC). Using a high polarity packing material, separation is based on the hydrophilic interactions of the sugars on the stationary phase surface typically using an eluent with a majority of polar organic solvent.

HILIC columns with amino functional groups are often used to prevent the separation of anomers; however, one drawback of the amino functional group on the packing material is the low recovery rate of reducing sugars, such as glucose and mannose. Reducing sugars are able to adhere to the packing material through the formation of a Schiff base, and must be hydrolyzed with acid for removal.

Shodex introduces the VG-50 4D column packed with a durable polymer based packing material modified with chemically stable tertiary amino functional groups. Shodex VG-50 4D is suitable for saccharide analysis, has demonstrated the separation of reducing sugars, and has been compared to two competitor silica-base amino columns.

Experimental Conditions

The analysis of four saccharides is accomplished with Shodex VG-50 4D (4.6 mm I.D. × 150 mm, 5 µm), a polymer-based amino

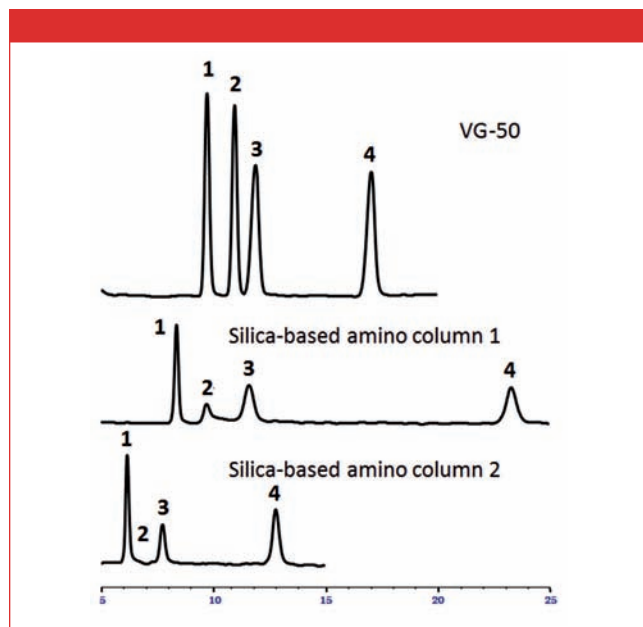


Figure 1: The analysis of sugars using VG-50 4D and two silica-based amino columns. Column: Shodex VG-50 4D (top) and silica-based amino columns (middle and bottom); column temperature: 40 °C; injection volume: 5 µL; eluent: CH₃CN/H₂O = 80/20; flow rate: 0.4 mL/min for VG-50 4D and 1.0 mL/min for silica columns. Detector: Shodex RI. Sample: 1. fructose, 2. mannose, 3. glucose, and 4. sucrose.

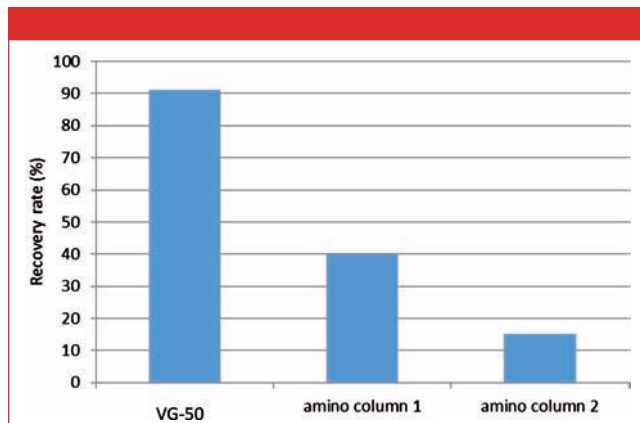


Figure 2: Comparison of Shodex VG-50 4D and silica-based amino columns demonstrating recovery ratio of mannose. Recovery ratio = (peak area mannose)/(peak area sucrose).

HILIC column. The separation is compared to the same analysis using two silica-based amino columns under the same conditions. Column temperature was 40 °C and flow rate was 0.4 mL/min for the VG-50 4D analysis and 1.0 mL/min for the silica-based amino analyses. Eluent conditions are 80% acetonitrile in water. Injection volume of 5 µL of 5 mg/mL of each sugar was used for each experiment. The HPLC system was coupled with RI detector.

Results

The saccharides, fructose, mannose, glucose, and sucrose, were analyzed successfully by HPLC and RI detection with VG-50 4D and two silica-based amino columns (Figure 1). A comparison of the recovery ratio of mannose, a reducing sugar, in relation to sucrose, a nonreducing sugar, is demonstrated in Figure 2. Shodex VG-50 4D demonstrates baseline separation of the four sugars with an elution volume of 20 min and allows up to 90% recovery rate of mannose. Silica-based amino column 1 shows baseline separation of the four compounds. However, the recovery ratio of reducing sugars is 40%. Silica-based amino column 2 cannot separate the four sugars, and the recovery ratio is less than 20%.

Conclusions

Shodex VG-50 4D, a polymer-based hydrophilic interaction (HILIC) chromatography column suitable for saccharide analysis, has demonstrated the recovery ratio of reducing sugars compared with silica-based amino columns.



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Determination of Highly Polar Pesticide Residues in Food of Plant Origin, by an Automated QuPPE Solution

Tyler Trent* and Rick Jordan†,

*Teledyne Tekmar and †Pacific Agricultural Laboratory

In the global market for fresh fruit, the United States is a major exporter of apples and orange fruits to various countries. However, exporters need to be mindful of the maximum residue levels (MRLs) of pesticides and food additives for various countries. Since many of these fruits need to be shipped over long distances, a coating is applied post-harvest with glazing agents to shield their surfaces from insects and fungi. Three components of these coatings, morpholine, diethanolamine (DEA), and triethanolamine (TEA) have come under scrutiny by the European Union (EU) due to the toxicological concerns with these compounds inherent tendency to oxidize to nitroso compounds which are known carcinogens.

Currently there is a validated method in the EU “Quick Method for Analysis of Residues of numerous Highly Polar Pesticides in Food of Plant Origin involving Simultaneous Extraction with Methanol and LC-MS/MS Determination (QuPPE-Method)” to determine morpholine, DEA, and TEA residues on waxed commodities.

The aim of this project is to evaluate the performance and versatility of the AutoMate-Q40 for the extraction of highly polar compounds using the QuPPE-Method M7.

Sample Preparation and Results

For the automated QuPPE extraction, 15 g of sample was weighed into a centrifuge tube and placed inside the AutoMate-Q40 for the extraction. The system will add 2.0–2.4 mL of water depending on the moisture content of the sample. Following the addition of water, the AutoMate-Q40 will add 15.0 mL of extraction solvent (1.0% acetic acid in methanol). Once the liquid additions were complete the sample was shaken vigorously and centrifuged for 1 min by the AutoMate-Q40. Then, 4 mL of the completed extract was transferred to the final extraction tube using the automated pipetting function. The extract was then removed

from the system and introduced into the LC-MS-MS for analysis.

Automating the QuPPE extraction enables fast, easy, reliable, and more highly reproducible extractions. The AutoMate-Q40 improves the repeatability and consistency between samples.

Table I shows that when using the AutoMate-Q40 to extract highly polar residues from apples and oranges recoveries ranging from 70.00% to 116.0%. Table I also shows that the results have excellent precision ranging from 1.72% to 14.29%.

Conclusion

Automation of the QuPPE extraction method produced reliable results for the spiked samples, which compared favorably with those from the existing manual procedure. There was excellent agreement between results for the analysis of samples prepared manually and using the AutoMate-Q40.3 Automating this procedure led to improved repeatability, a reduction in the likelihood of human error, and the potential for significant labor savings.

References

- (1) M. Hengel, R. Jordan, and W. Maguire, “Development and Validation of a Standard Method for the Determination of Morpholine Residues in Fruit Commodities by Liquid Chromatography-Mass Spectrometry,” *J. Agric. Food Chem.*, **62** (17), pp. 3697–3701 (2014). <http://pubs.acs.org/doi/full/10.1021/jf500734b>

Acknowledgments

A special thanks to Rick Jordan and Pacific Agricultural Laboratory for their time, support, and for conducting this analysis for Teledyne Tekmar.



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Table I: Analytical results for the QuPPE M7 method

Matrix	Fortification Level mg/kg	Morpholine			Diethanolamine (DEA)			Triethanolamine (TEA)		
		Recovery %	RSD %	MDL*	Recovery %	RSD %	MDL*	Recovery %	RSD %	MDL*
Apple	0.10	113.00%	6.20		106.00%	5.66		102.00%	6.86	
	0.05	116.00%	1.72		112.00%	1.79		108.00%	1.79	
	0.01	100.00%	10.0	0.0039	100.00%	10.00	0.0036	110.00%	9.09	0.0043
Orange	0.10	96.00%	4.17		83.00%	4.82				
	0.05	100.00%	4.00		82.00%	2.44				
	0.01	110.00%	9.09	0.0034	70.00%	14.29	0.0037			

Calculated Method Detection Limit (MDL) = Stdev of concentration found in 0.01 mg/kg spike * Student t99 (n-1), for seven reps (n=7), Student t= 3.143.

Amino Acid Analysis According to European Pharmacopoeia 8.0

Pickering Laboratories

The *European Pharmacopoeia* (*Ph. Eur.*) defines requirements for the qualitative and quantitative composition of medicines, as well as the tests to be carried out on medicines and on substances and materials used in their production.

It covers active substances, excipients, and preparations of chemical, animal, human or herbal origin, homeopathic preparations and homeopathic stocks, antibiotics, as well as dosage forms and containers. It also includes tests on biologicals, blood and plasma derivatives, vaccines, and radiopharmaceutical preparations. The *European Pharmacopoeia* and its requirements are legally binding in the member states of the European Pharmacopoeia Convention and the European Union.

All manufacturers of medicines or substances for pharmaceutical use therefore must apply the *Ph. Eur.* quality standards in order to be able to market and use these products in Europe.

Amino acids analysis can be used for:

- Identification tests on biopharmaceutical active ingredients (such as peptides, proteins) by means of amino acids composition analysis;
- Impurities and related substances determination on active pharmaceutical ingredients (APIs) (such as free amino acids) and intermediates;
- Single or total amino acids quantification in drug products, including markers determination in complex matrixes (such as phytopharmaceuticals).

The following *Ph. Eur.* monographs have already officially introduced the amino acid analysis method with post-column ninhydrin derivatization as the analytical procedure required for the determination of the ninhydrin-positive substances, and additional papers are expected to be published in upcoming months:

Cysteine HCl Monohydrate 01/2014:0895, Isoleucine 07/2013:0770, Leucine 07/2013:0771, Lysine HCl 07/2013:0930, Serine 01/2014:0788, Proline 01/2014:0785, Threonine 01/2014:1049, Valine 01/2014:0796, Arginine 07/2014:0806

Pickering Laboratories, Inc. offers a complete solution for amino acids analysis according to *European Pharmacopoeia* 8.0. This includes the Pinnacle PCX post-column derivatization instrument, analytical columns and GARDs, buffers and Trione® Ninhydrin reagent. The Pinnacle PCX is capable of performing column temperature gradients that allow easily modified conditions and improved run times and amino acids separations. The methods presented in this application note were optimized to comply with system suitability requirements of Pharmacopoeia 8.0 methods.

Each Pharmacopoeia monograph describes the preparation of the test and reference solutions specific for each amino acid. The solutions are used for calculations of percentage contents, impu-

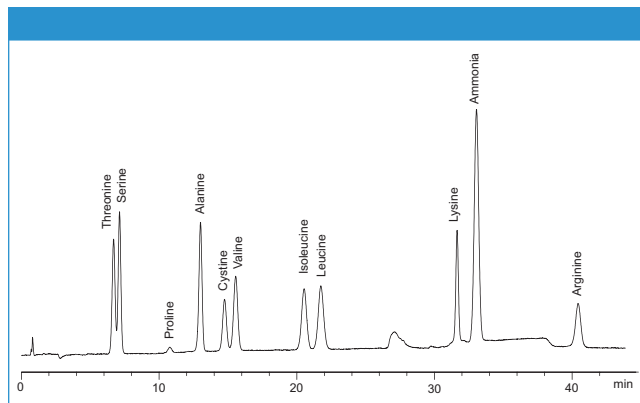


Figure 1: Sodium chromatogram of amino acids analyzed using Pharmacopoeia 8.0 methods (3 µg/mL each, 50 µL injection).

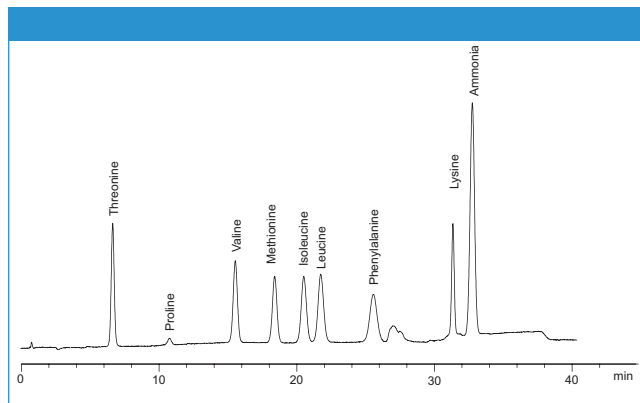


Figure 2: Sodium chromatogram of alternative amino acids analyzed using Pharmacopoeia 8.0 methods (3 µg/mL each, 50 µL injection).

rity levels as well as parameters of system suitability. Resolution of 1.5 is required between leucine and isoleucine peaks.

For all amino acids, except cysteine, sodium-based and lithium-based methods are available. For cysteine analysis, only lithium-based methods are suitable. Sodium-based methods have shorter run times and are preferable for all amino acids except cysteine.

Equipment:

Quaternary HPLC pump, autosampler, UV-vis detector
Pinnacle PCX post-column derivatization system

Analytical Columns and Eluants:

For sodium-based methods: High-efficiency sodium cation-exchange column, 4.6 × 110 mm, catalog number 1154110T. Eluants: Na315, Na425, Na640, RG011

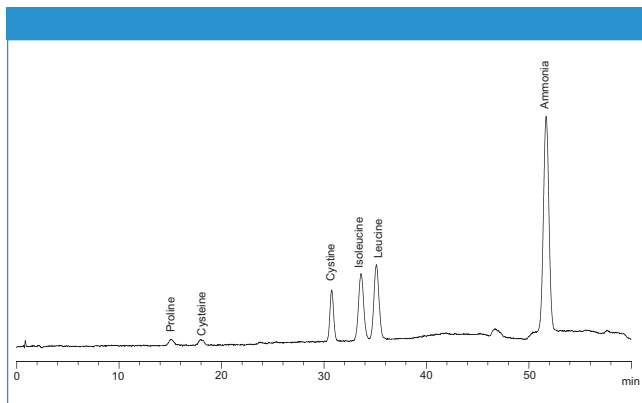


Figure 3: Lithium chromatogram of amino acids used as reference solutions for cysteine analysis (3 µg/mL each, 50 µL injection).

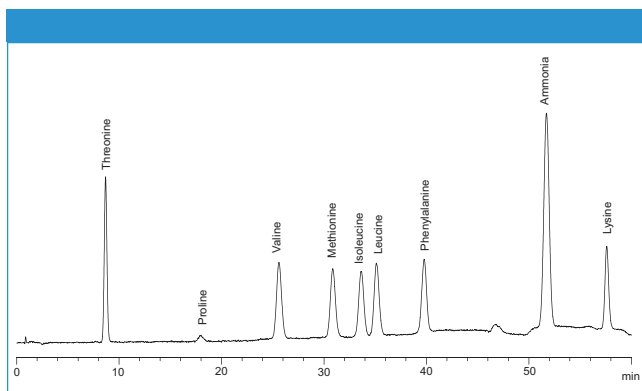


Figure 4: Lithium chromatogram of amino acids analyzed using Pharmacopoeia 8.0 methods (3 µg/mL each, 50 µL injection).

For lithium-based methods: High-efficiency lithium cation-exchange column, 4.6 × 75 mm, catalog number 0354675T. Eluants: 1700-1125, Li365, Li375, RG003

Post-Column Reagent

Trione® Ninhydrin Reagent

To make it easier to start using Pickering Laboratories methods, we offer chemistry kits that include: analytical column, GARD, buffers, and reagents for amino acids analysis. All parts of the kit could be ordered individually if needed. Please contact Pickering Laboratories if you have any questions regarding this application.

Pickering Laboratories will keep updating its Pharmacopoeia 8.0 methods as new monographs are released. Please contact support@pickeringlabs.com for the latest methods and chromatograms.



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Accurate Pain Management Analysis in Under 5 Min on Raptor™ Biphenyl Superficially Porous Particle LC Columns

Sharon Lupo, Ty Kahler, and Paul Connolly, Restek Corporation

Pain management LC analyses can be difficult to optimize due to the limited selectivity of C18 and phenyl-hexyl phases. In contrast, the selectivity of Raptor™ Biphenyl superficially porous particle (SPP) LC columns provides complete resolution of isobaric pain medications with a total cycle time of 5 min.

Accurate, reliable analysis of pain medications is a key component in monitoring appropriate medical use and preventing drug diversion and abuse. As the demand for fast, multicomponent methods grows, LC–MS–MS methods are increasingly desired for pain management and therapeutic drug monitoring due to the low detection limits that can be achieved with this highly sensitive and selective technique. However, despite the selectivity offered by mass spectrometry, hydrophilic matrix components can still interfere with early-eluting drug compounds resulting in ion suppression. In addition, isobaric pairs must be chromatographically separated for positive identification. The need for highly selective and accurate methods makes LC column selection critical.

While C18 and phenyl-hexyl phases are frequently used for bioanalytical LC–MS–MS applications, Restek's Biphenyl phase offers better aromatic retention and selectivity for pharmaceutical and drug-like compounds, giving it a significant advantage over other phases for the analysis of pain management medications or other drugs of abuse. The Biphenyl phase, originally developed a decade ago by Restek, has recently been combined with Raptor™ SPP ("core-shell") silica particles to allow for faster separations without the need for expensive UHPLC instrumentation. Here, we demonstrate the fast, selective separation of commonly tested pain drugs that can be achieved using the new Raptor™ SPP Biphenyl LC column.

Table I: Mobile phase gradient

Time (min)	Flow (mL/min)	%A	%B
0.00	0.6	90	10
1.50	0.6	55	45
2.50	0.6	0	100
3.70	0.6	0	100
3.71	0.6	90	10
5.00	0.6	90	10

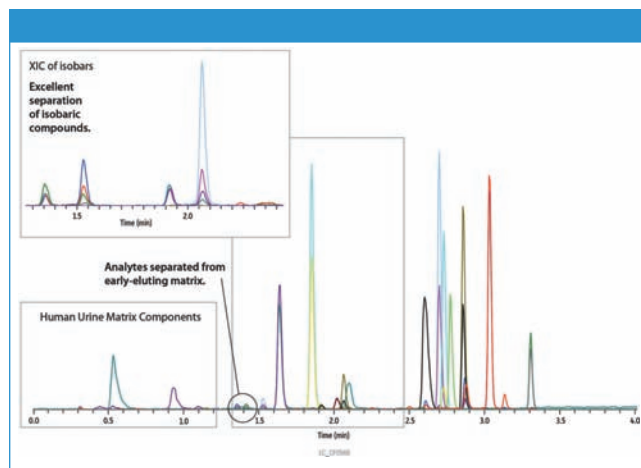


Figure 1: Baseline resolution of isobaric pain management drugs in sub-5-min runs on the Raptor™ Biphenyl column.

Experimental Conditions

A standard containing multiple pain management drugs was prepared in blank human urine and diluted with mobile phase as follows, urine:mobile phase A:mobile phase B (17:76:7). The final concentration for all analytes was 10 ng/mL except for lorazepam, which was 100 ng/mL. Samples were then analyzed by LC–MS–MS using an AB SCIEX API 4000™ MS–MS in ESI+ mode. Chromatographic conditions, retention times, and mass transitions are presented here and in Tables I and II:

Column: Raptor™ Biphenyl, 50 mm × 3.0 mm i.d. × 2.7 μm
Sample: Fortified urine
Inj. vol.: 10 μL
Inj. temp.: 30 °C
Mobile phase A: Water + 0.1% formic acid
Mobile phase B: Methanol + 0.1% formic acid

Results

As shown in Figure 1, 18 commonly tested pain management drugs were analyzed with the last compound eluting in less than 3.5 min, giving a total cycle time of 5 min on Restek's Raptor™ SPP Biphenyl LC column. Analyte retention times are presented in Table II. Important isobaric pairs (morphine/hydromorphone and codeine/hydrocodone) were completely resolved and eluted as symmetrical peaks, allowing accurate identification and integration. In addition, early-eluting compounds such as morphine, oxycodone, and

Table II: Analyte retention times and transitions

Peaks	t_R (min)	Precursor Ion	Product Ion 1	Product Ion 2
Morphine*	1.34	286.2	152.3	165.3
Oxymorphone	1.40	302.1	227.3	198.2
Hydromorphone*	1.52	286.1	185.3	128.2
Amphetamine	1.62	136.0	91.3	119.2
Methamphetamine	1.84	150.0	91.2	119.3
Codeine*	1.91	300.2	165.4	153.2
Oxycodone	2.02	316.1	241.3	256.4
Hydrocodone*	2.06	300.1	199.3	128.3
Norbuprenorphine	2.59	414.1	83.4	101.0
Meprobamate	2.61	219.0	158.4	97.2
Fentanyl	2.70	337.2	188.4	105.2
Buprenorphine	2.70	468.3	396.4	414.5
Flurazepam	2.73	388.2	315.2	288.3
Sufentanil	2.77	387.2	238.5	111.3
Methadone	2.86	310.2	265.3	105.3
Carisoprodol	2.87	261.2	176.3	158.1
Lorazepam	3.03	321.0	275.4	303.1
Diazepam	3.31	285.1	193.2	153.9

*An extracted ion chromatogram (XIC) of these isobars is presented in the inset of Figure 1.

hydromorphone are separated from hydrophilic matrix interferences, resulting in decreased ion-suppression and increased sensitivity. Similar analyses on C18 and phenyl-hexyl columns often exhibit poor peak shape and resolution (for example, peak tailing between closely eluting isobars), which makes identification and accurate quantification more difficult.

Conclusions

Complete separation of critical pain management drug analytes from hydrophilic matrix components and isobaric interferences was achieved using the new Raptor™ SPP Biphenyl LC column in less than 5 min. The fast, complete separations produced in this method allow accurate quantification of pain management drugs and support increased sample throughput and improved lab productivity.

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CAPCELL CORE ADME S2.7, a Novel Adamantyl Modified Stationary Phase, Alternative to C18 for Improved Separation of Metabolites

Y. Hiroe, M. Seio, H. Arai, X. Xin, and T. Kanda, Frontier Science Business Division, Shiseido Co. Ltd.

CAPCELL CORE ADME S2.7 (ADME) is a column packed with a novel stationary phase of superficially porous silica (core shell particle) modified by adamantyl functional groups. ADME has separation characteristics with a balance between moderate hydrophobicity and extra-high surface polarity, which is completely different from the conventional C18. Therefore, ADME could provide separation patterns and separation improvement previously unavailable in C18 with increased retention to highly polar compounds. It is also expected as a significant alternative to C18 column for improved separation of samples having diverse polarities such as metabolites and the matrix in real samples.

The C18 column is the most popular one used in reversed-phase chromatography, but has difficulty in retaining highly polar compounds. As a solution, high polar columns applicable in 100% aqueous mobile phase (1) or changing the separation mode to hydrophilic interaction chromatography (HILIC) (2) accompanied with re-setup of the analysis condition are considerable, but troublesome or with limitations. Meanwhile, in recent years, comprehensive analysis of metabolites has raised the need for simultaneous analysis of multi-component comprising highly polar metabolites and highly hydrophobic compounds before metabolism not only in pharmacokinetics fields.

Shiseido succeeded in the development of CAPCELL CORE ADME S2.7 (ADME), a novel stationary phase with surface modification of Adamantyl functional group which is completely different with the C18. ADME is easy to use just as a general reversed-phase column since its functional group is also alkyl group only with changed structure.

In this study, the characteristics of CAPCELL CORE ADME S2.7 (ADME) and the separation behavior of highly polar compounds with ADME column were evaluated.

Experimental

Basic feature of CAPCELL CORE ADME S2.7

1. Determination of separation parameters

CAPCELL CORE ADME S2.7 (ADME) is a column packed with a novel particle with 1.7- μm solid core and 0.5- μm porous layer modified with adamantyl functional group (Figure 1). The parameters of hydrophobicity and surface polarity were determined with ADME by the resolution factors of toluene with benzoate, and toluene with methyl benzoate, respectively (3).

2. Comparison of separation characteristics

The effect of separation parameters on retention and separation were determined by comparison of the analysis of ethynyl estradiol and its metabolites with CAPCELL CORE C18 S2.7 and CAPCELL CORE AQ S2.7, respectively.

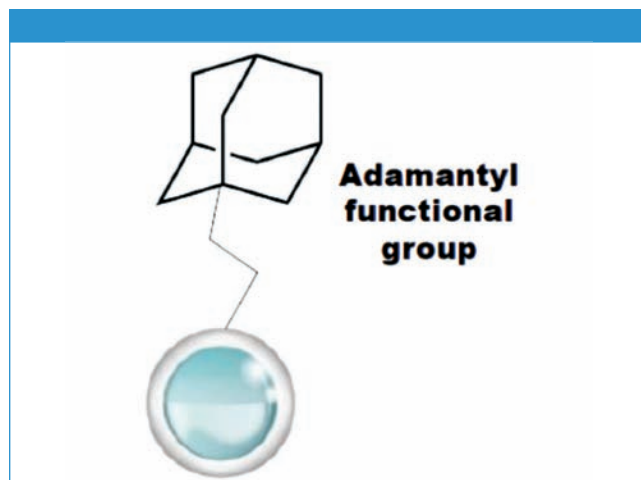


Figure 1: Superficially porous particle modified by adamantyl functional groups.

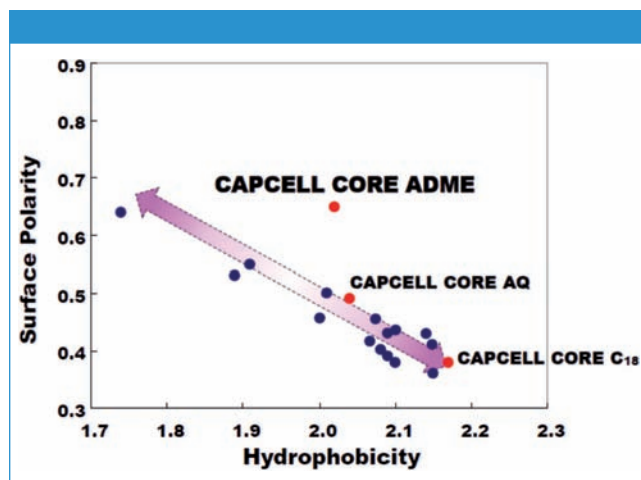


Figure 2: Parameter map of separation.

3. Application of CAPCELL CORE ADME S2.7

Ethynyl estradiol, its relative substances and conjugated metabolites were analyzed with CAPCELL CORE ADME S2.7.

Results and Discussion

Basic feature of CAPCELL CORE ADME S2.7

(1) Determination of separation parameters

In Figure 2, the parameters of hydrophobicity and surface polarity of CAPCELL CORE ADME S2.7 derived from the resolution factors of toluene with benzoate, and toluene with methyl benzoate were plotted on the parameter graph including CAPCELL CORE C18 S2.7,

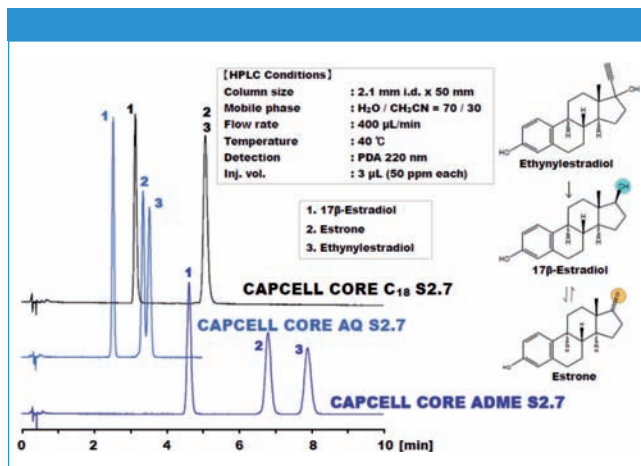


Figure 3: Chromatograms of nithynyl estradiol and its metabolites.

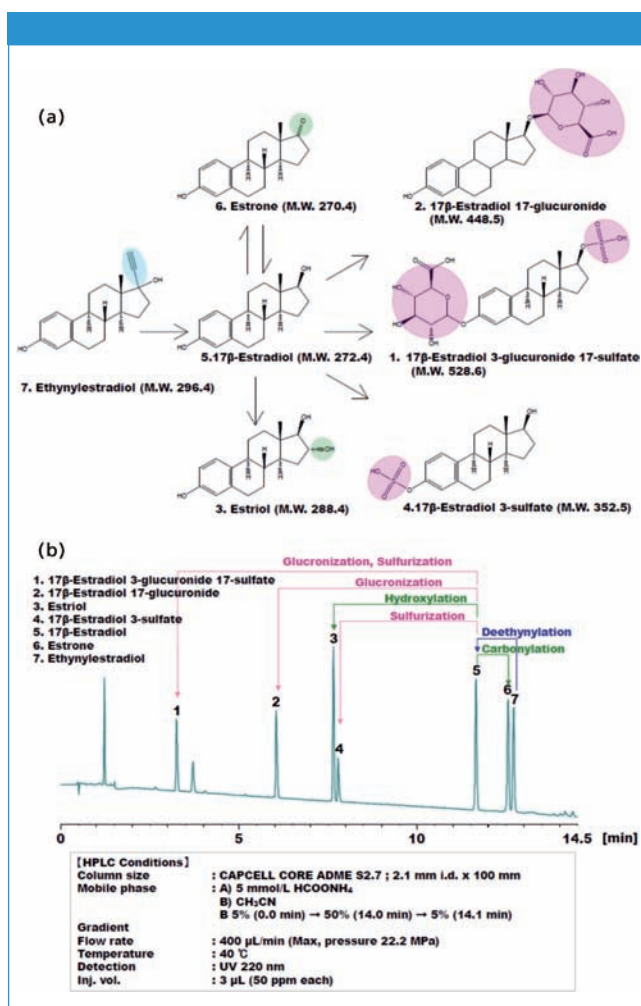


Figure 4: (a) Metabolic pathway of ethynylestradiol. (b) Simultaneous analysis of nithynyl estradiol relative substances.

CAPCELL CORE AQ S2.7, and other reversed-phase columns. In the parameter map, ADME is positioned at moderate hydrophobicity

and extra-high surface polarity.

(2) Comparison of separation characteristics

As shown in the chromatograms in Figure 3, CAPCELL CORE ADME S2.7 performed with improved separation compared with more hydrophobic CAPCELL CORE C18 S2.7 and similarly hydrophilic CAPCELL CORE AQ S2.7. Therefore, it is suggested that not only the hydrophobicity but also the high surface polarity of ADME provided effect on the separation of ethynyl estradiol and its metabolites (17-β estradiol and estrone).

Application of CAPCELL CORE ADME S2.7

Ethynyl estradiol and its conjugated metabolites containing glycoside were analyzed under gradient condition with CAPCELL CORE ADME S2.7 (Figure 4b). It is clear that the metabolites of ethynyl estradiol, conjugate of sulfuric acid and glucuronic acid were certainly separated in the simultaneous analysis of multi-components comprising unchanged ethynyl estradiol and its relative metabolites in different stages (Figure 4a).

Conclusion

CAPCELL CORE ADME S2.7 is a novel core-shell type column with adamantyl-functional group modified stationary phase and provides improved separation to highly polar metabolites which is previously unavailable by the conventional C18 columns. ADME is easy to use as a reversed-phase column and expected as a significant alternative solution to C18 in the simultaneous analysis of metabolites and the separation of matrix in a real sample.

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Separation of Macrocyclic Lactones

Diamond Analytics

Avermectins are a series of 16-membered macrocyclic lactone derivatives that are used extensively in animal and crop protection. They occur in nature and can be produced as fermentation by-products by the micro-organism, *streptomyces avermitilis*. Examples of well-known avermectins and derived products include ivermectin, eprinomectin, selamectin, doramectin, and abamectin. All the avermectins have high anthelmintic and insecticidal properties even at low dose levels and residues of these veterinary drug components reach the environment through manufacturing and animal waste and may potentially affect terrestrial and aquatic life forms.

Several crop protection companies have strong interest in the synthesis, production, and analysis of these compounds. Therefore, there exists a need to develop a fast and efficient analytical method capable of determining avermectin residues on animals, in food, and in the environment. The HPLC method presented here is fast and efficient and can be used for residual analysis as well as for quality control of avermectins in drug formulations. The peaks produced are sharp resulting in high sensitivity.

The mixture can be baseline separated using gradient elution with an MS-compatible mobile phase containing acetonitrile, methanol, and water. What is even more interesting is that the four avermectin products considered and up to 20 separate degradation products could be identified.

HPLC Conditions

Column name:	FLARE C18 MM
Column dimensions:	4.6 × 150 mm (15698-14-2, 3.6 µm, 120 Å)
HPLC system:	Agilent 1200
Inj. vol. and conc.:	5.0 µL, ~0.2 mg/mL of each major analyte in MeOH
Detection:	UV at 244 nm
Flow rate:	1.0 mL/min
Solvents:	A: 50 mL ACN, 100 mL MeOH, 850 mL H ₂ O; B: 600 mL ACN, 200 mL MeOH, 200 mL H ₂ O
Gradient:	0.00 min, 10% B; 10.00 min, 90% B; 10.01 min, 10% B; 18.00 min, 10% B
Temperature:	35 °C

The FLARE C18 MM column is manufactured with 3.6 µm diamond core-shell particles that have 120 Å pores. The particle geometry and tight particle size distribution contribute to high packing density and column efficiency with reduced plate height, *h*, of ~2. The columns come in various lengths and diameters and are compatible

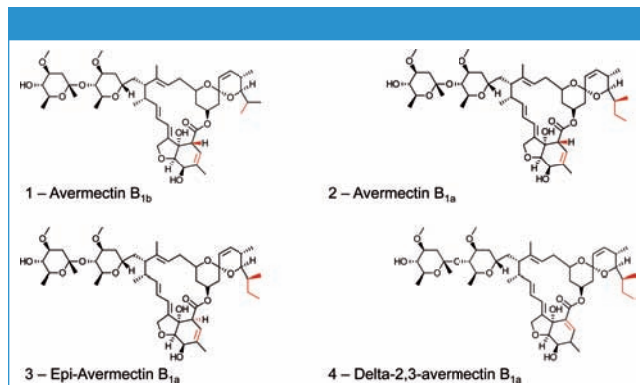


Figure1: Structures of selected macrocyclic lactones.

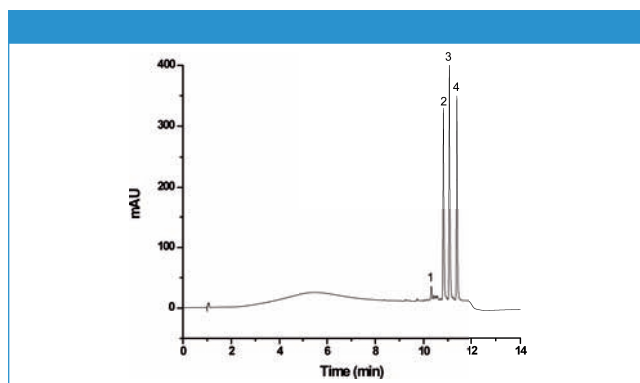


Figure2: Separation of macrocyclic lactones using FLARE C18 MM column.

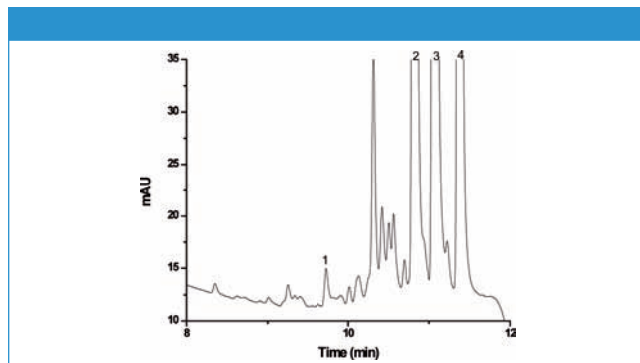


Figure3: Exploded view showing peaks between 8 and 12 min.

with 100% aqueous to 100% organic solvents. They are pH and temperature stable and available to ship worldwide.



Diamond Analytics

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Characterization of PLGA Using SEC–MALS–VIS

Wyatt Technology Corporation

Poly(lactic-co-glycolic acid) (PLGA) is a copolymer based on glycolic acid and lactic acid. The two monomer units are linked together by ester linkages and form linear polyester chains. The obtained product is biodegradable and biocompatible, and it is approved by the Food and Drug Administration (FDA) for production of various therapeutic devices as well as for drug delivery applications. The properties of PLGA can be tuned by the ratio of the two monomers and by its molar mass distribution.

The characterization of PLGA by means of conventional size-exclusion chromatography (SEC) is problematic because of the lack of suitable calibration standards. In addition, the linear polyester structure can be modified by the addition of small amounts of poly-functional monomer to obtain branched chains of differing degrees of branching. The degree of branching becomes an additional parameter that can be used to adjust PLGA properties—all of which renders conventional column calibration an inadequate analytical technique.

In this application note, two commercially available samples were analyzed by SEC coupled to a multi-angle light scattering (MALS) detector (HELEOS), a refractive index detector (Optilab rEX), and a viscosity (VIS) detector (ViscoStar). The ViscoStar was used in order to discover additional information about the molecular structure of the analyzed polymers. In addition to molar mass distributions, the SEC–MALS–VIS system yields the relationship between intrinsic viscosity and molar mass (Mark-Houwink plot) that can provide deep insight into the molecular structure of the polymers being analyzed.

In Figure 1, the molar mass distributions are given as differential distribution plots. As seen from the plots, the two samples span markedly different molar mass ranges. The Mark-Houwink plots of the two samples are shown in Figure 2 together with the plot of linear polystyrene that is shown simply for the sake of comparison. The slope of the Mark-Houwink plot of the linear polystyrene is 0.71, a typical value for linear random coils in thermodynamically good solvents. The slope of the red sample roughly corresponds to a linear structure as well. However, there is a slight indication of deviation from linearity at the region of high molar masses that may indicate the presence of branched molecules. The Mark-Houwink plot of the blue sample is curved. Curvature of the Mark-Houwink plot generally reveals branching. In addition, the slope of the higher molar mass portion of the Mark-Houwink plot of 0.48 suggests significant branching.

SEC–MALS–VIS is an excellent method for the characterization of PLGA polyesters as it has the ability to determine not only the molar mass distribution, but to reveal subtle differences in PLGAs molecular structure.

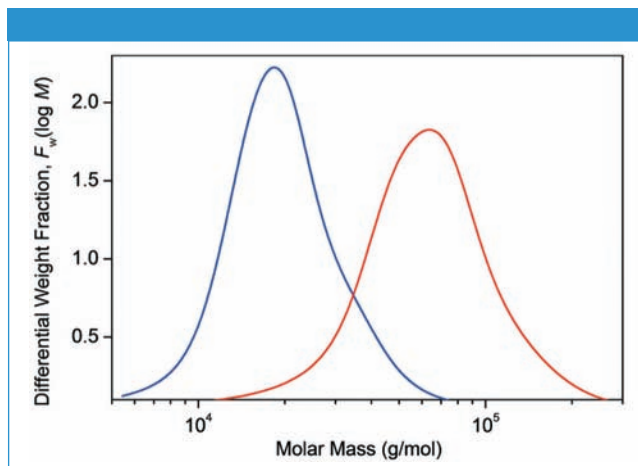


Figure 1: Differential molar mass distribution curves of two PLGA samples.

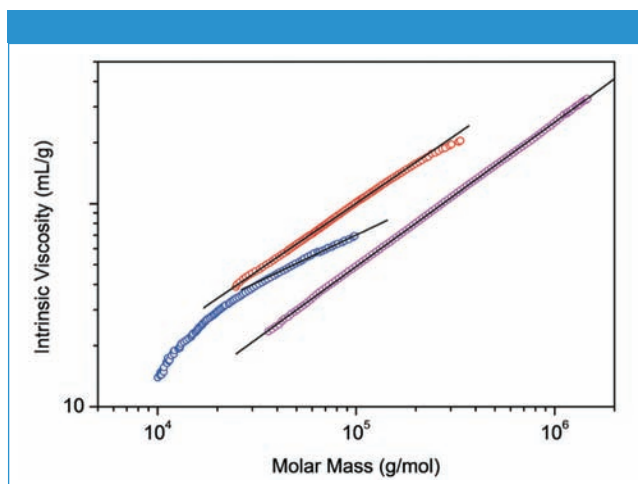


Figure 2: Mark-Houwink plots of two samples of PLGA (red and blue) and linear polystyrene (magenta). The lines are linear extrapolations of the data.



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Structure-Molecular Weight Relationships of Synthetic Polymers

Malvern Instruments Ltd.

The physical properties and behavior of polymers depends strongly on the properties of the polymer molecules themselves. This in turn means that the polymer properties will also have an impact on the properties of finished products made from or containing polymers. The molecular weight and molecular weight distribution, molecular size, intrinsic viscosity, and structure all affect how the polymer material will behave. Gel permeation or size-exclusion chromatography (GPC/SEC) allows you to investigate the properties of synthetic polymers and generate a Mark-Houwink (MH) plot, a representation of the structure-molecular weight relationship generated by plotting molecular weight (MW) against intrinsic viscosity (IV).

Polystyrene (PS), polymethylmethacrylate (PMMA), polycarbonate (PC), and polyvinylchloride (PVC) were all separated using two Viscotek T6000M columns and a mobile phase of stabilized THF. An OMNISEC system with UV, RI, light scattering, and viscometer detectors was used to measure the molecular weight, intrinsic viscosity, and structure of each synthetic polymer.

Results and Discussion

Table I summarizes the calculated data for all four samples showing the absolute molecular weights calculated directly from the light scattering detector using the appropriate dn/dc for each of the polymer types. The system gives values for the measured IV and hydrodynamic radius (Rh) as well as other parameters including the average MH and log k values. From this we can clearly see the differences between the polymer types. For instance, PVC has the highest IVw value but the second lowest Mw value. However, looking at the average values for broad polymers does not always give a complete picture of their differences so it is better to look at the structure across the whole MW distribution using the MH plot.

The Mark-Houwink plot of all four polymers shown in Figure 1 clearly shows the structural differences. PMMA is lowest on the plot indicating that it has the highest density in solution, with PS being slightly higher and therefore less dense than the PMMA. PVC and PC

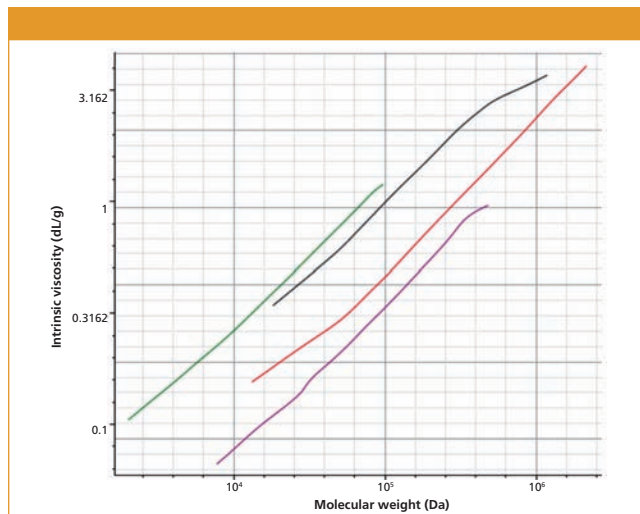


Figure 1: Mark-Houwink overlay of all four polymer samples showing the structural differences. Polystyrene (red), PMMA (purple), polycarbonate (green), and PVC (black).

have intrinsic viscosities that are much higher than PS at any given MW, indicating more open and less dense structures.

Conclusion

These four common polymers were analyzed fully and in detail using the OMNISEC GPC/SEC system. The combination of absolute MW values and IV data allows simple access to the powerful MH plot. The data this plot delivers is of utmost importance to all polymer chemists who want to understand polymer differences or trends in terms of molecular weight and structural changes independently from each other. In practice, these differences might be visualized and compared between different batches of polymer or production processes to identify samples with the most desired molecular properties such as branching level or molecular weight distribution. These, in turn, will have the desired bulk properties in their final uses.



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Table I: Calculated data for all four synthetic polymers

Parameter	Samples			
	Polyvinylchloride	Polystyrene	Polymethylmethacrylate	Polycarbonate
Retention volume (mL)	17.220	17.363	18.343	18.850
Mn (Da)	81,930	108,300	57,323	10,189
Mw (Da)	202,910	250,890	101,840	27,270
Mz (Da)	563,930	428,720	147,950	41,603
PD (Mw/Mn)	2.477	2.317	1.777	2.676
Intrinsic viscosity (dL/g)	1.370	0.834	0.327	0.486
Rhw (nm)	15.281	14.103	7.776	5.672

THE APPLICATION NOTEBOOK

Call for Application Notes

LCGC is planning to publish the next issue of *The Application Notebook* special supplement in September. The publication will include vendor application notes that describe techniques and applications of all forms of chromatography and capillary electrophoresis that are of immediate interest to users in industry, academia, and government. If your company is interested in participating in these special supplements, contact:

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It is important that each company's material fit within the allotted space. The editors cannot be responsible for substantial editing or handling of application notes that deviate from the following guidelines:

Each application note page should be no more than 500 words in length and should follow the following format.

Format

- **Title:** short, specific, and clear
- **Abstract:** brief, one- or two-sentence abstract
- **Introduction**
- **Experimental Conditions**
- **Results**
- **Conclusions**
- **References**
- **Two graphic elements:** one is the company logo; the other may be a sample chromatogram, figure, or table
- **The company's full mailing address, telephone number, fax number, and Internet address**

All text will be published in accordance with LCGC's style to maintain uniformity throughout the issue. It also will be checked for grammatical accuracy, although the content will not be edited. Text should be sent in electronic format, preferably using Microsoft Word.

Figures

Refer to photographs, line drawings, and graphs in the text using arabic numerals in consecutive order (Figure 1, etc.). Company logos, line drawings, graphs, and charts must be professionally rendered and submitted as

.TIF or .EPS files with a minimum resolution of 300 dpi. Lines of chromatograms must be heavy enough to remain legible after reduction. Provide peak labels and identification. Provide figure captions as part of the text, each identified by its proper number and title. If you wish to submit a figure or chromatogram, please follow the format of the sample provided below.

Tables

Each table should be typed as part of the main text document. Refer to tables in the text by Roman numerals in consecutive order (Table I, etc.). Every table and each column within the table must have an appropriate heading. Table number and title must be placed in a continuous heading above the data presented. If you wish to submit a table, please follow the format of the sample provided below.

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Literature citations must be indicated by arabic numerals in parentheses. List cited references at the end in the order of their appearance. Use the following format for references:

(1) T.L. Einmann and C. Champaign, *Science* **387**, 922–930 (1981).

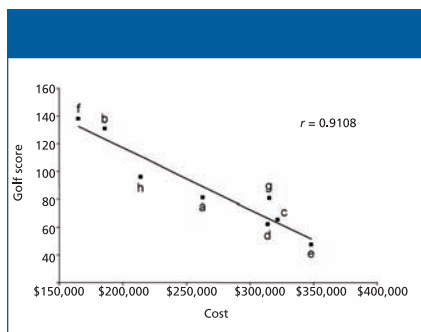


Figure 1: Chromatograms obtained using the conditions under which the ion suppression problem was originally discovered. The ion suppression trace is shown on the bottom. Column: 75 mm × 4.6 mm ODS-3; mobile-phase A: 0.05% heptafluorobutyric acid in water; mobile-phase B: 0.05% heptafluorobutyric acid in acetonitrile; gradient: 5–30% B in 4 min. Peaks: 1 = metabolite, 2 = internal standard, 3 = parent drug.

Table I: Factor levels used in the designs

Factor	Nominal value	Lower level (–1)	Upper level (+1)
Gradient profile	1	0	2
Column temperature (°C)	40	38	42
Buffer concentration	40	36	44
Mobile-phase buffer pH	5	4.8	5.2
Detection wavelength (nm)	446	441	451
Triethylamine (%)	0.23	0.21	0.25
Dimethylformamide	10	9.5	10.5

The deadline for submitting application notes for the September issue of *The Application Notebook* is:

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Mike Tessalone at (732) 346-3016, Ed Fantuzzi at (732) 346-3015, Stephanie Shaffer at (774) 249-1890, or Lizzy Thomas at (574) 276-2941.

Synthetic Rubbers: Polybutadiene

Wyatt Technology

Synthetic elastomers have replaced natural rubber to an astonishing degree, and account for more than 70% of the rubber used today. In the United States alone, 5 million tons of synthetic rubber are produced annually. The principal synthetic rubber elastomer is a copolymer of butadiene and styrene. The latex form of rubber and synthetic elastomers has applications in carpet and gloves, and coagulated latex is used for the production of tires and mechanical goods. It is of critical importance to know the absolute molar mass and its distribution, as well as to gain insight into the conformation of synthetic rubber—which are indicative of the product's end-use performance.

Typically, polystyrene standards are used to estimate the molar masses of these polymers in SEC experiments, but by using a DAWN or miniDAWN multi-angle light scattering (MALS) detector, standards and column calibration are no longer needed. Here, the absolute molar mass and polydispersity, as well as the rms radius of two synthetic rubber samples were measured directly using SEC combined with a DAWN.

The synthetic rubber samples were analyzed in toluene, and Wyatt Technology's Optilab was used as the refractive index detector for the SEC line. The refractive index increment dn/dc of polybutadiene in toluene is relatively low, and the dn/dc value of the butadiene/styrene copolymer in toluene increases with the ratio of styrene present. In Figure 1, the molar mass and its distribution are determined absolutely—without using any standards or calibration routines.

In addition to the weight-average molar mass and the polydispersity, the DAWN can also determine the shape of the polymer by

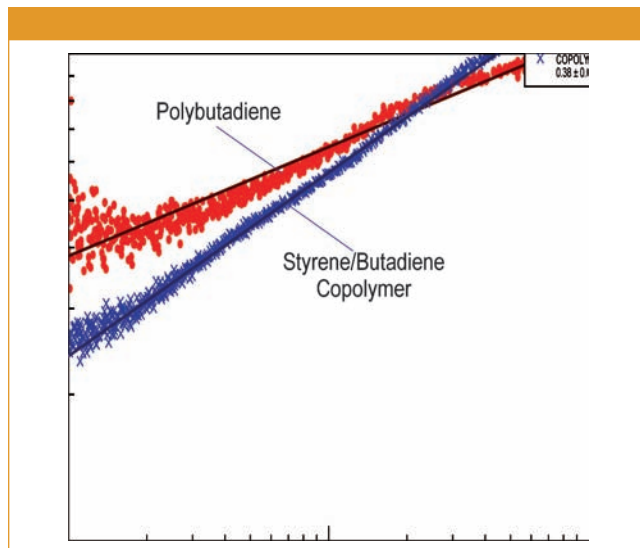


Figure 2: RMS radius versus molar mass ("conformation plot") for the two samples. The slope is indicative of the conformation (rod, coil, or sphere) of the molecule.

measuring the rms radius directly at each elution volume. It is well-known that butadiene forms highly branched polymers and that styrene forms linear polymers—and this is revealed by the DAWN.

Figure 2 shows a logarithmic plot of the molar mass versus the radius of the two synthetic rubber samples. The slope of such a plot is indicative of the shape of a polymer. A slope between 0.5 and 0.6 is usually found for linear polymers with a random coil conformation, while spheres have a slope of approximately 0.3. The values obtained for polybutadiene (0.25) and for the butadiene/styrene copolymer (0.38) indicate that this polybutadiene is more branched than the styrene/butadiene copolymer.

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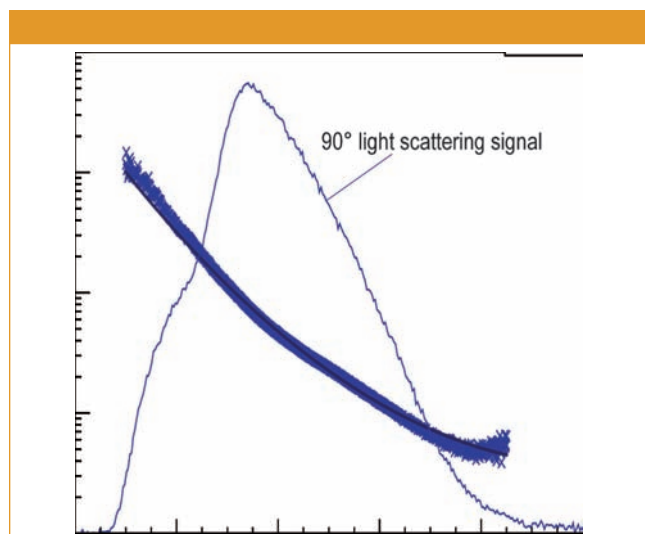


Figure 1: Molar mass versus elution time for the synthetic rubber sample superimposed upon the signal from one of the light scattering detector channels.

Extraction of Drugs of Abuse in Urine Using Solid Phase Extraction

FMS

The term drugs of abuse (DOA) can be applied to a wide variety of natural and synthetic compounds prevalent in human culture. Many rapidly pass through the human body, while others can persist for extended periods. Screening for traces of these has become common place by analyzing human urine, either by LC–MS or by GC–MS through derivatization of the parent compounds.

Solid phase extraction (SPE) provides an ideal extraction media for DOA compounds, as it can be performed with small sample amounts with rapid turnaround. The utilization of automated SPE platforms (EconoTrace SPE, FMS Inc.) can greatly assist production laboratories generate large volumes of samples in relative short periods of time.

Consumables

- 150 mg 6 cc DOA mixed mode SPE cartridges
- Acetonitrile, LC–MS grade or equivalent
- Methanol, LC–MS grade or equivalent
- Toluene, pesticide grade or equivalent
- HPLC grade water
- 6N HCL
- Ammonium hydroxide
- Native and labeled DOA standards acquired from Cambridge Isotopes Laboratories.

Sample/Reagent Prep

1. 2 mL urine samples diluted to 5 mL DI water, pH adjusted to 2 with HCL.

SPE Procedure

1. Condition cartridges with 5 mL methanol at 2 mL per min
2. Condition cartridges with 5 mL DI water at 2 mL per min
3. Sample loaded across cartridge at 2.5 mL per min
4. Cartridge washed with 5 mL water
5. Cartridge washed with 5 mL methanol
6. Cartridges dried with nitrogen for 20 min
7. Cartridges eluted with 4 mL 3:2 methanol – acetonitrile with 5% ammonium hydroxide
8. Cartridges allowed to soak for 3 min
9. Cartridges eluted with 3 mL 3:2 methanol – acetonitrile with 5% ammonium hydroxide
10. Remaining elution solvent N₂ purged to collection vials

SuperVap

1. Preheat temperature: 35 °C
2. Evap mode: 8 psi nitrogen with sensor
3. Extracts reduced to 1 mL volume

SuperVap Vial Evaporator

At 1 mL, GC vials removed from SuperVap tubes and transferred to vial evaporator. Extracts taken to dryness at 2 psi, ambient temperature. Extracts reconstituted with recovery standard in initial mobile phase.

Analytical Conditions

Waters Acquity H-Class UPLC

Column: Waters BEH C₁₈, 2.1 × 100 mm, 1.7 µm

Column temperature: 30 °C

Solvent A: 0.1% formic acid in MilliQ water

Solvent B: 0.1% formic acid in acetonitrile

Xevo TQD mass spectrometer conditions:

Ionization mode:

Acquisition mode:

Capillary voltage:

Collision energy (eV):

Cone voltage (V):

Data:

Time (min)	Flow (mL/min)	%A	%B	Curve
0	0.4	98	2	
6	0.4	52.8	47.2	6
7	0.4	52.8	47.2	6
7.5	0.4	98	2	6
8.5	0.4	98	2	6

Compound	Percent Recovery	RSD
Amphetamine	111	10.2
Buprenorphine	114	8
Cocaine	103	6.2
Codeine	100	13.6
Fentanyl	89	11
Hydrocodone	114	14.2
Hydromorphone	92	11.2
MDMA	97	9.8
Merperidine	97	7.2
Methadone	90	8.6
Methamphetamine	100	7.2
Naloxone	106	6.1
Naltrexone	97	6.9
Oxycodone	94	6.8
Oxymorphone	96	0.5
PCP	102	4.8
Tramadol	94	2.8

ESI+ and ESI-

MRM

1.5 kV

optimized for individual compounds

optimized for individual compounds

acquisition and analysis using Mass

Lynx v.4.1 software

Conclusions

Extraction efficiencies for DOA compounds in human urine matrix showed excellent precision using the mixed mode cartridge. Between replicates, RPDs were below 15% for all analytes with most falling below 10%. With the easy operation, limited sample prep and excellent performance the FMS, Inc. EconoTrace SPE demonstrates to be an ideal fit for DOA screening of human urine.



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Improved Retention for Polar Organic Acids with Unique Multi-Mode Columns in Reversed Phase Conditions

Robert Puryear, Piotr Macech, Eric Wendt, and Itaru Yazawa, Imtakt USA

The challenging retention of polar organic acids in reversed phase conditions is accomplished using a unique multi-mode stationary phase. Retention characteristics are compared with a standard ODS stationary phase highlighting the value of additional selectivities in development of methods for organic acids.

Analysis of organic acids in a mixture has been challenging due to the high polarity of these compounds. Typically, organic acids are analyzed with both polar and nonpolar analytes using reversed phase ODS columns. This can be difficult because their hydrophobic moieties may not differ from analyte to analyte or their polar functional groups may override this interaction. Therefore, the use of a multi-mode column like Scherzo SM-C18, which utilizes weak anion and weak cation exchange ligands in addition to hydrophobic ODS ligands provides additional ionic retention mechanisms, allowing for superior retention of organic acids.

Experimental Conditions

A 4 μ L sample at 3.8 to 6.2 μ g concentration was injected onto a Unison UK-C18 and a Scherzo SM-C18 column (250 $\times$ 3.0 mm, 3 μ). Eluent was analyzed using ELSD detection (spray chamber 19 $^{\circ}$ C, drift tube 43 $^{\circ}$ C). Further experimental details are provided as an insert in Figure 1.

Results and Discussion

Direct comparison of two different reversed phase columns shows improved retention of organic acids on Scherzo SM-C18 versus a traditional ODS column. In this experiment the variables were reduced to only a change in stationary phase where column dimension, particle size, and gradient conditions were identical.

In this case, retention of all organic acids tested showed an increase in retention time except for succinic acid, which was reduced by 1.7

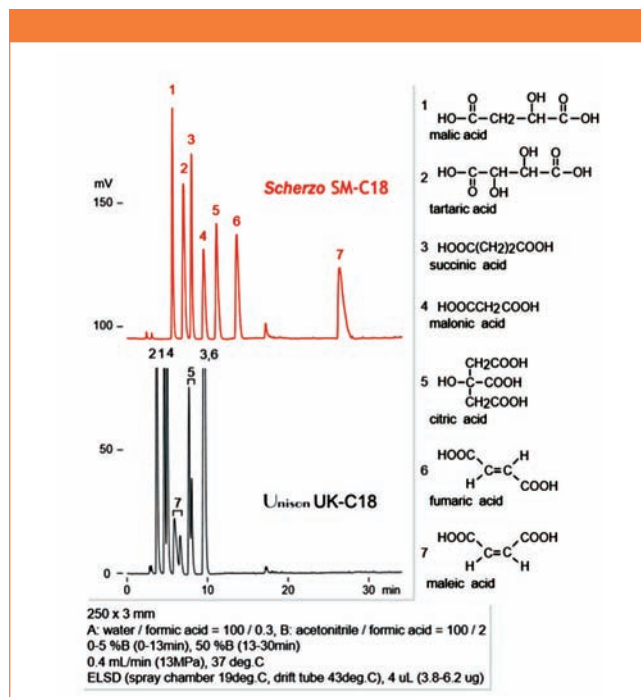


Figure 1: Comparison of retention for several organic acids on a Scherzo SM-C18 multi-mode versus traditional ODS stationary phase.

min. This could be due to a dominance of hydrophobic retention for this molecule, which would be slightly reduced in Scherzo compared to the Unison column because of a change in carbon load. The most dramatic increase was seen with maleic acid which had an additional 20.4 min of retention on Scherzo SM-C18. This could be due to the fact that maleic acid has a high dipole moment charge (7.2 debyes) because of its asymmetric cis configuration. This is in contrast to its trans-isomer fumaric acid which is symmetrical and therefore no initial dipole moment.

Overall peak shape was also improved particularly for citric and maleic acid, where these analytes showed split peaks (7.6 and 7.9, 5.9 and 6.5, respectively) on a traditional ODS column.

Conclusion

In summary, use of a multi-mode stationary phase like Scherzo SM-C18 has several advantages over traditional ODS column in the analysis of organic acids. With better retention of these polar compounds, this column can provide better resolution in reversed phase conditions by the addition of ion exchange retention mechanisms. These additional selectivities make it possible to separate organic acids in reverse phase conditions which previously was not possible by traditional ODS stationary phases.



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Table I: Change in retention time (delta RT). Values listed are in minutes.

	Unison UK-C18	Scherzo SM-C18	Delta RT
malic acid (1)	4.5	5.6	1.1
tartaric acid (2)	3.7	6.8	3.1
succinic acid (3)	9.5	7.8	-1.7
malonic acid (4)	5	9.4	4.4
citric acid (5)	7.6	11.1	3.5
fumaric acid (6)	9.5	13.6	4.1
maleic acid (7)	5.9	26.3	20.4

Make Your Unknowns, "Known" with High Resolution Mass Spectrometry

Elizabeth Humston-Fulmer and Lorne Fell,
Life Science and Chemical Analysis Centre, LECO Corporation

The combination of high performance mass spectrometry and comprehensive two-dimensional GC definitively takes unknown components and unambiguously identifies an important odor component in a perfume sample.

GC-MS has been an important tool in the perfume industry for decades, providing robust characterization of samples for quality control, new product development, and even competitive analysis. These capabilities can be extended with the addition of a complementary separation dimension with GC×GC, and with a high performance mass spectrometer to better separate and identify components in a perfume sample.

Experimental Conditions

Samples were diluted and injected for analysis on three instrument platforms; Pegasus® HT GC-TOFMS, Pegasus 4D GC×GC-TOFMS, and Pegasus GC-HRT 4D.

Results

Figure 1 demonstrates improved confidence in the identification of an important odor analyte by improving chromatographic separation and adding accurate mass detection with GC×GC-HR-TOFMS. The analyte was found (through first dimension retention and spectral consistency) and compared in GC-TOFMS, GC×GC-TOFMS, and GC×GC-HR-TOFMS data. The spectrum for iso-eugenol in the GC-TOFMS experiment matched very poorly to the library standard at less than

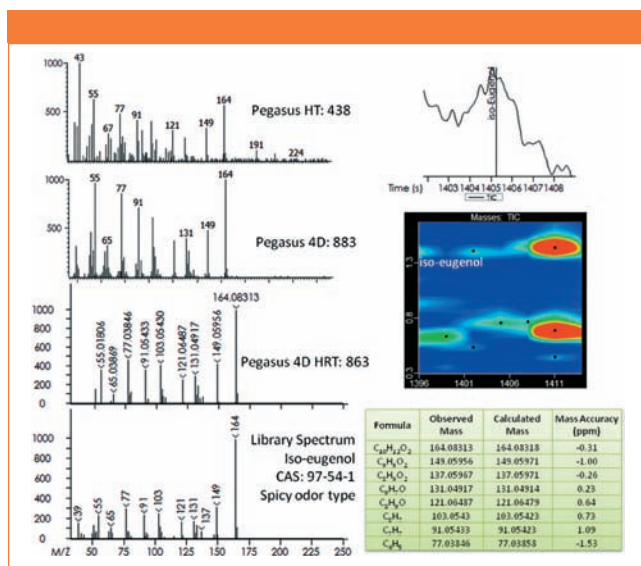


Figure 1: A comparison of spectra obtained from Pegasus HT, Pegasus 4D, and the Pegasus GC-HRT 4D for a particular component in a perfume sample.

Table 1: Experimental conditions (parameters specific to GC×GC italicized)

Gas Chromatograph	Agilent 7890 with Dual Stage Quad Jet Modulator and MPS2 Autosampler
Injection	1 µL splitless with inlet @ 250 °C
Carrier gas	He @ 1.0 mL/min, <i>Pressure Corrected Constant Flow</i>
Column one	Rxi-5ms, 30 m × 0.25 mm i.d. × 0.25 µm coating (Restek)
Column two	Rxi-17SilMS, 1.20 m × 0.25 mm × 0.25 µm coating (Restek)
Temperature program	2 min at 40 °C, ramped 5 °C/min to 280 °C, held 10 min <i>Secondary oven maintained +15 °C relative to primary oven</i>
Modulation	3 s with temperature maintained +15 °C relative to secondary oven
Transfer line	250 °C
Mass Spectrometer	LECO Pegasus® HT or Pegasus GC-HRT 4D
Ion source temperature	250 °C
Mass range	33-500 <i>m/z</i>
Acquisition rate	20 spectra/s for GC or 100 spectra/s for GC×GC

50% similarity. Without additional information this would likely be considered an unknown. When analyzed by GC×GC-TOFMS, the similarity to the library spectrum improved to over 80%, which was primarily related to the improved chromatographic separation. There are several *m/z* in the low mass region (<100 *m/z*) that are observed in the GC-TOFMS spectrum, and are absent in the GC×GC-TOFMS spectrum, that were attributed to coeluting analytes in the first dimension separation which were chromatographically separated in the second dimension. GC×GC provided both a cleaner iso-eugenol spectrum and the ability to detect the coeluting analytes. Confirmation of identification is often pursued with the analysis of standards, which is not always possible due to availability or cost restrictions. The same level of confidence can be attained by pairing a high resolution mass spectrometer with the GC×GC separation. The GC×GC-HR-TOFMS spectrum improved the confidence with a library similarity of over 80%, mass accuracy of the molecular ion (RDBE = 5) of < 1 ppm, and mass accuracy of fragments across the mass range of less than 1 ppm, on average.

Conclusion

High performance mass spectrometry and comprehensive GC (GC×GC) provides improved confidence of the presence of iso-eugenol, a characteristic spicy odor component in a perfume sample that was difficult to identify with GC-TOFMS.



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Replacing Stacked Ring Reflectron Lenses with Segmented Monolithic Resistive Glass

Photonis, USA

Reflectron lenses are used in time-of-flight (TOF) mass spectrometers to create an electrostatic field to alter ion flow, providing for a longer flight path and therefore greater resolution. Current reflectron-type TOF-MS instruments use complex multi-piece stacked ring assemblies that require time consuming assembly and cleaning processes. They also require the use of a voltage divider in each layer to control the electric field.

Segmented monolithic lenses made from resistive glass can replace traditional stacked ring reflectron lenses currently used in mass spectrometers. Resistive glass tubes are designed to guide charged particles by generating a highly uniform electric field. Resistive glass products are composed of a proprietary lead silicate glass that has been specially processed to create an integral semi-conductive layer on the surface.

Prior studies detailing the use of a resistive glass reflectron tube in an orthogonal TOF system showed the resistive glass tube had

lower FWHM values, indicating a better energy focus, while comparative spectra between a traditional stacked ring assembly and the resistive glass tube were nearly identical (1).

An additional patent was granted to Photonis in 2012 for the manufacture of varied, nonlinear electric fields in resistive glass tubes. This new manufacturing capability enables instrument designers to produce nonlinear and dynamic fields within the lens for better instrument performance. Axial lines can be applied for use as a collision cell, or rings can be applied for use as a segmented reflectron lens.

A reflectron lens made with resistive glass provides a solid assembly replacement for a stacked ring assembly yet provides the same ability to alter ion flow. This innovation is a form-fit-function replacement for the multi-piece stacked ring assemblies, while providing the ability to manipulate the electric field inside the resistive glass tube.

The single piece assembly also greatly simplifies the cleaning and assembly process currently required for stacked ring lenses. Resistive glass can be easily cleaned with water, acetone, methanol, or IPA without degrading performance, and is resistant to scratches from light to moderate abrasions.

References

- (1) S. Ritzau, B. Laprade, S. Mrotek, and R. Leffingwell, "A Direct Comparison of a Resistive Glass and Stacked-Ring Reflectron," Burle Electro-Optics, ASMS 2006.



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Enhanced-Fluidity Liquid Chromatography: Connecting the Dots Between Supercritical Fluid Chromatography, Conventional Subcritical Fluid Chromatography, and HPLC

Enhanced-fluidity liquids are organic solvents or organic–aqueous solvents mixed with high proportions of liquefied gases, such as carbon dioxide. These subcritical solvents share the positive attributes of supercritical fluids (fast diffusion rates and low viscosities) and the positive attributes of commonly used liquids (high solvent strength). These solvent properties provide enhanced efficiency for reversed-phase, conventional normal-phase, hydrophilic-interaction, and size-exclusion chromatography. The capabilities of enhanced-fluidity liquid chromatography for highly polar compounds is described and the value of using the entire continuum of 0–100% carbon dioxide solvent systems is discussed in terms of changes in mobile-phase properties and applications.

Susan V. Olesik

Enanced-fluidity liquids (EFL) are mixtures of conventional liquids to which dissolved gases, such as carbon dioxide, are added. Our group coined this term in 1991 to explain the fact that these solvents possess fluidity (inverse of viscosity) that is markedly higher than typical liquids (1). This was at a time when supercritical fluid chromatography (SFC) was gaining interest. These mixtures are described as gas expanded liquids (GXLs) in the chemical engineering literature (2), but both terms are merely descriptive of the physical phenomena involved in producing the mixture. The addition of a liquefied gas to a conventional liquid causes considerable volume expansion of the mixture, and the fluidity of mixture increases substantially. Today, much of the work in SFC is actually performed under subcritical fluid conditions, meaning the separations are being performed below the critical point of the solvent. Enhanced-fluidity liquids are indeed subcritical solvents, but enhanced-fluidity liquid chromatography (EFLC) uses a smaller proportion of liquefied gas in the mobile phase than in typical subcritical fluid chromatography, which is 0–50% organic modifier. In EFLC, conditions from 100% to 50% conventional liquid combined with a liquefied gas are used in the mixtures. Therefore, by combining conventional subcritical fluid chromatography with EFLC, the entire solvent range of 0–100%

organic solvent is spanned. This solvent range is highly useful for chromatographic applications. Like in SFC, EFLC typically uses carbon dioxide as the liquefied gas, but other liquefied gases have also been evaluated, such as fluoroform (3,4).

Optimization of Chromatography

In high performance liquid chromatography (HPLC), the fastest separations are achieved when working at the highest possible pressure of the chromatographic instrument. Using reduced (dimensionless) plate height, h , and reduced velocity, v , as defined in equations 1 and 2, the Knox-Saleem equation (equation 3) illustrates a linear relationship between retention separation time, t_o , and viscosity,

$$h = H/d_p \quad [1]$$

$$v = ud_p/D_m \quad [2]$$

$$t_o = \Phi h_{\min}^2 N_{\text{req}}^2 \eta / \Delta P^2 \quad [3]$$

$$N/t_R = D_m / d_p^2 (v/h)(1/1 + k) \quad [4]$$

where H is plate height, u is linear velocity, d_p is the particle size of the packing, D_m is the diffusion coefficient

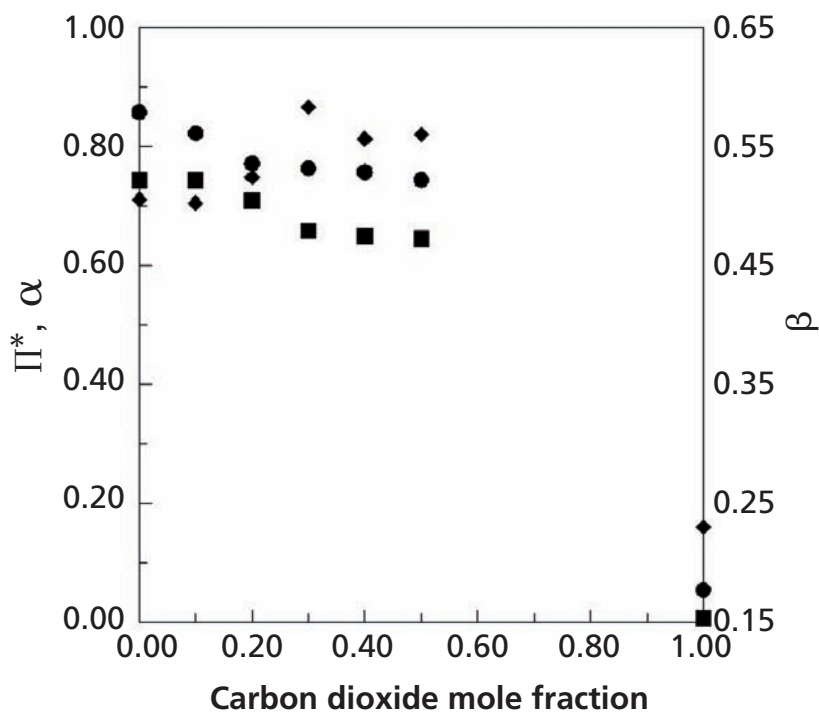


Figure 1: Variation of Kamlet-Taft solvatochromic parameters for methanol-water-carbon dioxide mixtures as a function of added carbon dioxide with the mole ratio of methanol-water held at 2.3 at 25 °C and 172 bar (● = A, ◆ = E, ■ = π*). Data adapted from reference 11.

is the pressure drop across the column, and t_R is the retention time (5). Equation 3 assumes an optimized chromatographic system in terms of column length, particle size, and flow rate. Assuming maintenance of operating conditions near these conditions, a substantial decrease in time of analysis is observed by lowering the viscosity of the mobile phase. Finally, Guiochon and others illustrated that the separation power, N/t_R (6,7) can be increased by increasing the mobile-phase diffusion coefficient assuming the other parameters do not vary much and the entire separation is functioning at the optimum reduced velocity where k is the retention factor.

$$N/t_R = D_m/d_p^2(v/h)(1/1 + k) \quad [4]$$

Properties of EFL Mixtures Solvent Strength and Dielectric Constant

An interesting attribute of enhanced-fluidity liquid mixtures is that as much as 60 wt% liquefied gas can be added before considerable loss of solvent strength occurs (1,4,8). Mixtures of alcohols and carbon dioxide are robust in their solvent strength, particularly methanol-carbon dioxide mixtures. Aida and Inomato (9) studied the molecular structure of methanol-carbon dioxide mixtures using molecular dynamics (MD) simulations. Their study illustrated that up to 50 wt% carbon dioxide can be added to methanol before impacting the hydrogen bond (H-bond) network. However, below that percentage the H-bond network degraded. Their data and the solvent strength measurements correlate well. The dielectric constant of methanol-carbon dioxide mixtures (50 °C and 110 bar) decrease linearly with added carbon dioxide until 0.45 mole fraction methanol is added. Further addition of carbon dioxide to methanol decreases the dielectric constant, but at a slower rate. The dielectric constant with 0.60 mole fraction methanol was nearly half that of pure methanol (10).

Figure 1 shows the variation of solvent strength when carbon dioxide is

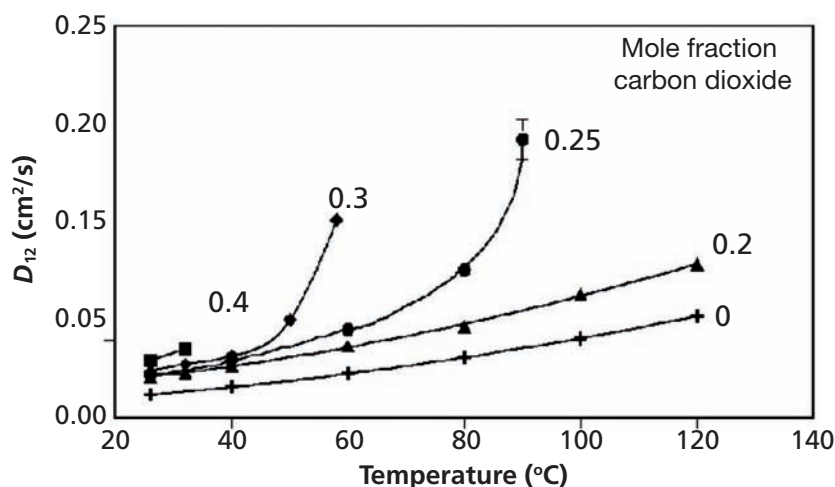


Figure 2: Variation of the diffusion coefficient of benzene at 138 bar in 0.70:0.30 methanol-water (+), 0.56:0.24:0.20 (▲), 0.52:0.23:0.25 (●), 0.49:0.21:0.30 (◆), 0.42:0.18:0.40 (■) methanol-water-carbon dioxide. Data adapted from reference 13.

of the analyte, N_{req} is the required chromatographic efficiency for a given separation, t_o is the retention

time (seconds), Φ is the dimensionless flow resistance parameter, η is the viscosity of the mobile phase, ΔP

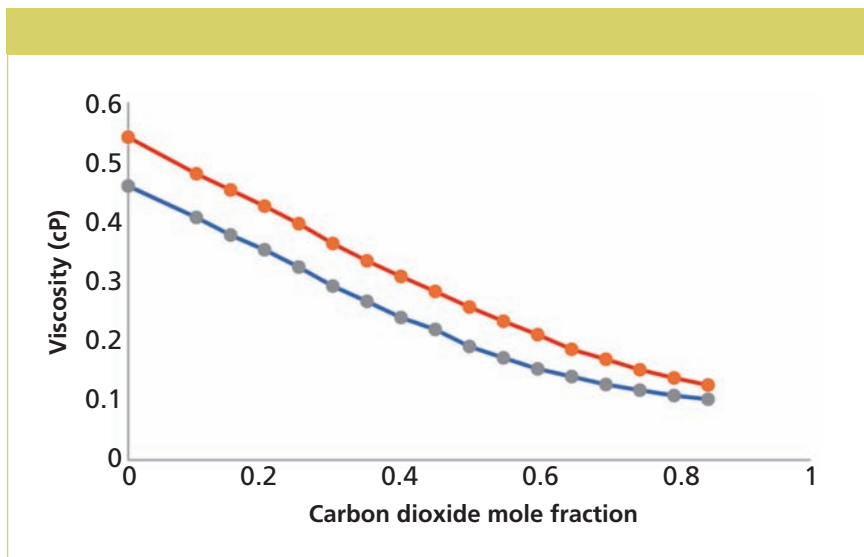


Figure 3: Variation of viscosity of a methanol–carbon dioxide mixture as a function of added carbon dioxide at 25 °C (red curve, pressure increased from 0 bar to 56.7 bar with increasing carbon dioxide) and 40 °C (blue curve, pressure increased from 1 bar to 76.7 bar with increasing carbon dioxide). Data adapted from reference 14.

added to a 0.70:0.30 mole ratio methanol–water mixture at 25 °C and 172 bar (11). Similarly, carbon dioxide can be added to other solvent systems with minimal loss in solvent strength. However, the unique attribute of these data is that the hydrogen bond basicity of the ternary mixtures appears to increase with added carbon dioxide.

Buffers

When high proportions of carbon dioxide are added to liquids, the solvents become weakly acidic unless other additives are included to control the pH. Buffers can be readily produced in these mixtures. We previously illustrated that buffers could be produced in methanol–water–carbon dioxide mixtures with pH values from 2.2 to 6.8 (12). The addition of carbon dioxide to methanol–water mixtures with a mole ratio of 69:31 produces a buffer with pH varying from 4.54 to 4.73 depending on the proportion of carbon dioxide added. The formation of carbonic acid and the presence of dissolved carbon dioxide provides the buffering components of this system. This buffer is very interesting because nonvolatile buffer additives are not necessary. The other interesting aspect of this study was that for the methanol–

water–carbon dioxide mixtures studied, increasing the pressure from 120 to 207 bar did not significantly impact the measured pH.

Diffusion Coefficients

Chromatographic band dispersion in liquid chromatography is typically highly influenced by the resistance to mass transfer between the mobile phase and the stationary phase, which is inversely proportional to the diffusion coefficient of the mobile phase. Therefore, for low band dispersion, high diffusion coefficients are desired. Diffusion coefficients of solutes in a number of enhanced fluidity liquids were measured. For the methanol–carbon dioxide mixtures at 25 °C and 172 bar, the diffusion coefficient of benzene increases by approximately 75% by adding carbon dioxide up to 50 mol% (1). With increasing carbon dioxide above 50 mol% the diffusion coefficients increase at a faster rate up to that of pure carbon dioxide.

The variation of solute diffusion coefficients in methanol–water–carbon dioxide mixtures are also nonideal (13). Temperatures in excess of 60 °C were needed to increase the diffusion coefficient of benzene to the same value as the addition of

0.30 mole fraction carbon dioxide (Figure 2). However, by increasing the temperature and adding carbon dioxide to this methanol–water mixture, the greatest benefit was observed. For a 0.70:0.30 mole ratio methanol–water mixture, the addition of 0.30 mole fraction carbon dioxide and an increase in temperature to 58 °C caused a ninefold increase in the diffusion coefficient of benzene.

Viscosity

Foster's group studied the change in viscosity in both methanol–carbon dioxide and ethanol–carbon dioxide mixtures. For methanol–carbon dioxide mixtures for pressures ranging from 12 bar to 78 bar and temperatures varying from 25 °C to 40 °C, the viscosity of the EFL decreased linearly to approximately 50% of the original viscosity from 0 to 50 mol% carbon dioxide (14). The viscosity continued to decrease with further addition of carbon dioxide, but not at the same rate. This change occurred at 50 mol% carbon dioxide for the entire temperature range. Ethanol–carbon dioxide mixtures were different; the viscosity decreased substantially with added carbon dioxide (15). However, the composition where the rate of change slowed varied with temperature. At 25 °C the reduction was linear up to 0.70 mole fraction carbon dioxide. For 30 °C, 35 °C, and 40 °C the rate of viscosity reduction slowed at 0.60, 0.45, and 0.25 mole fraction carbon dioxide, respectively. These data clearly show that the addition of carbon dioxide to conventional solvents will substantially impact the separation time.

Instrumentation

The instrumentation necessary to accomplish enhanced-fluidity chromatography is the same as that used in SFC with the condition that the software must allow use of more than 50% modifier. Alternatively, a conventional HPLC system can be used to deliver the organic solvents and a carbon dioxide-compatible

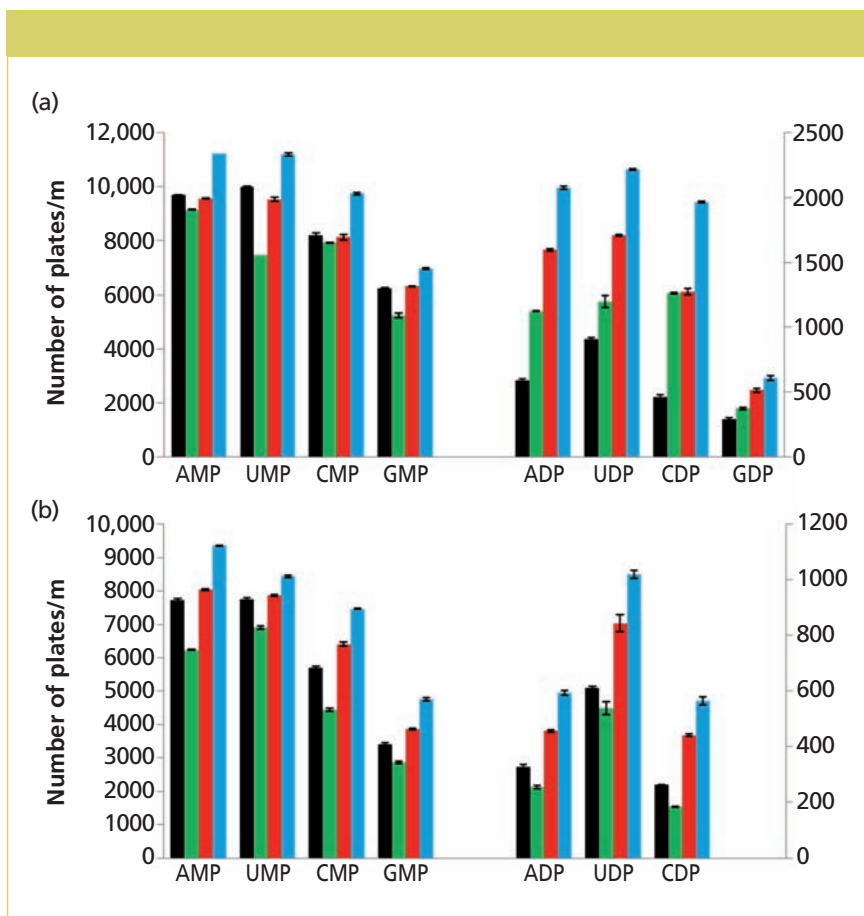


Figure 4: Effect of different bases on the efficiency of the nucleotides: (a) in LC and (b) with 0.1 mole fraction carbon dioxide added. Conditions: 5 mM of each base was added to a 75 mM ammonium phosphate solution, which was used to prepare the 90:10 (v/v) methanol–aqueous mobile phase. The y-axis on the left corresponds to the efficiency values for the monophosphate compounds, the axis on the right corresponds to the values for the diphosphate compounds. No base (■), TEA (■), DABCO (■), and DBN (■). Data adapted from reference 24.

pump and mixer can be added at the outlet of the HPLC pump to allow for the use of carbon dioxide in the mobile phase. With this setup, a restrictor (small diameter tubing) must be added at the exit of the detector to control the pressure and flow rate of the mobile phase. For example, Sandra (16) used an Agilent 1200 HPLC system combined with a separate carbon dioxide pump and mixer at the outlet of the Agilent 1200 HPLC pumps. A stainless steel flow restrictor (4 m × 0.12 mm i.d.) and a needle valve were placed at the detector outlet to control the flow.

Commercial SFC instrumentation can be used under subcritical conditions all the way to EFLC conditions as long as the pressure limit of the instrument or the software for the sol-

vent programming doesn't limit the mobile-phase conditions. (Note: These instruments use back-pressure regulators to control flow instead of fixed restrictors, as an example, an Agilent 1260 system can be used without modification for EFLC separations.)

Previous EFLC and a Range of Chromatographic Mechanisms

EFLC has been used effectively for reversed-phase, normal-phase, chiral, and size-exclusion modes of chromatography (17). In reversed-phase chromatography previous work has shown the separation time can be reduced by nearly half by adding carbon dioxide. Using normal-phase conditions for gradient polymer elution chromatography, Kawai also showed that the use of

carbon dioxide in the mobile phase provided the highest resolution for poly(styrene-*co*-methyl acrylate) mixtures compared to the use of conventional liquids (30). In addition, chiral separations are typically faster and more efficient than in HPLC or SFC (17). As summarized by Guiochon and Tarafder (18), higher mobile-phase velocities, longer columns, and finer particles can be used with EFLC compared to HPLC with conventional solvents. These attributes are shared with high-temperature HPLC and ultrahigh-pressure liquid chromatography (UHPLC). The higher diffusion coefficients cause higher optimum mobile-phase velocities compared to HPLC and lower resistance to mass transfer, which increases the overall efficiency.

Separation of Polar Compounds – Subcritical Conditions Including EFLC Conditions

There continues to be considerable interest in providing fast and efficient separations for highly polar compounds. Taylor and coworkers (19) have been studying the use of subcritical chromatography for the separation of polar compounds with encouraging results. For example, Zheng and colleagues (19) described the separation of polypeptides up to 40-mers using a 2-ethylpyridine bonded silica stationary phase and carbon dioxide–methanol mobile phases with trifluoroacetic acid as an additive in the methanol to suppress the deprotonation of carboxylic acid groups and protonate the peptide amino acid groups on the protein. A 5–50% methanol gradient was used. Electrospray mass spectra with high signal-to-noise ratios were obtained with this technique and a lower separation time was achieved as well when comparing the optimized protein separations under SFC and HPLC conditions. Ashraf-Khorassani and Taylor (20) also showed that alcohol–carbon dioxide mixtures with up to 5% water using gradient conditions up to 50% alcohol were effective in separating

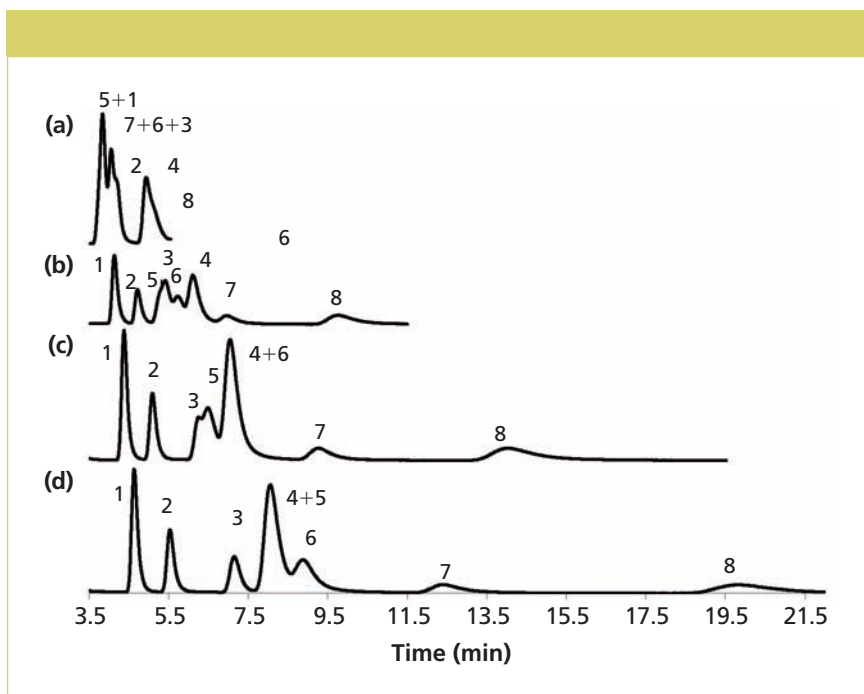


Figure 5: Separation of various nucleosides and nucleotides: (a) LC, (b) 0.15 mole fraction of carbon dioxide, (c) 0.20 mole fraction of carbon dioxide, and (d) 0.25 mole fraction of carbon dioxide. Mobile phase: 90:10 methanol–75 mM ammonium phosphate + 5 mM DBN. Peaks: 1 = adenosine, 2 = cytidine, 3 = uridine, 4 = guanosine, 5 = AMP, 6 = CMP, 7 = UMP, 8 = GMP. Data adapted from reference 24.

the nucleobases thymine, uracil, adenine, and cytosine.

Guillarme and coworkers (21) illustrated the value of subcritical fluid chromatography using a methanol–carbon dioxide mixture with 2–40% methanol at 40 °C and 150 bar with a 2-ethylpyridine column (100 mm × 3.0 mm, 1.7- μm d_p , Acquity UPC² BEH-2-EP, Waters) or an Acquity BEH Shield RP18 hybrid column (50 mm × 2.1 mm, 1.7- μm d_p , Waters) using 20 mM ammonium hydroxide in the mobile phase at a flow rate of 1.5 mL/min for the separation of highly polar compounds such as nucleobases and carbohydrates. A comparison of column types showed that gradient subcritical fluid chromatography could separate well at least 70% of the compounds studied with enhanced mass spectral sensitivity.

For chiral separations, Armstrong (22) evaluated subcritical conditions for chiral separations using macrocyclic glycopeptides. For the separation of polar analytes such as native amino acids using macrocyclic glycopeptides stationary phases, a combination of both acidic and basic modifiers and

subcritical conditions with 48% to nearly 70% methanol were quite effective in providing excellent separations.

Sandra's group (16) was the first to study the possibility of using EFLC conditions for hydrophilic-interaction chromatography (HILIC). In this study, using a silica column (250 mm × 4.6 mm, 5- μm d_p , Zorbax Rx–SIL, Agilent Technologies) the five nucleobases thymine, uracil, cytosine, guanine, and adenine were separated using 95:5 (v/v) ethanol–20 mM ammonium formate buffer at pH 3.0 combined with carbon dioxide. Increasing amounts of carbon dioxide increased the elution window and the separations were similar to those obtaining using acetonitrile–water mobile phases. Plate counts of 16,200 and 19,000 were obtained for 3 mL/min and 0.9 mL/min conditions.

Our group studied EFLC–HILIC separation of RNA nucleosides (adenosine [A], uridine [U], cytidine [C], and guanosine [G]) using EFLC mobile phases under isocratic conditions (23). Using a 150 mm × 4.6 mm, 3- μm d_p Tosoh amide column and a 90:10 methanol–20 mM acetate buffer

mobile phase, the addition of increasing proportions of carbon dioxide caused increased retention with a slight increase in band dispersion for the chromatographic bands. For example, the addition of 0.2 mole fraction carbon dioxide causes changes in the retention factor for A, C, U, and G of 72%, 66%, 97%, and 138%, respectively. With 20 mol% carbon dioxide, a separation time of 15 min was possible with resolution values >4 for all pairs. Alternatively, under optimized HPLC conditions with the same column using a 90:10 acetonitrile–20 mM acetate buffer, a complete separation was achieved in approximately 50 min.

To attempt separations of even more polar compounds, the separation of mono-, di-, and triphosphorylated nucleosides were studied (24). These compounds are typically separated using affinity chromatography, ion-exchange, or electrophoresis (25–27). A 150 mm × 4.6 mm, 3.5- μm d_p Waters X-bridge amide column was used with 90:10 methanol–aqueous mobile phases buffered with ammonium phosphate. Often, phosphorylated compounds have poor peak shapes because of strong tailing in a broad range of chromatographic retention mechanisms using silica as a support. Phosphorylated compounds are strong hydrogen bond bases that interact with free silanols on a silica column through very strong hydrogen bonding interactions (28). The slow kinetics of desorption are the likely cause of the peak asymmetry. Strong bases such as triethylamine (TEA) are often added to the mobile phase to compete with the interactions with the free silanols (29). Two bases that are widely used in organic synthesis and described as superbases because of their strong basicity in both aqueous and organic solvents were also considered and compared to TEA: 1,4-diazabicyclo [2.2.2] octane (DABCO) and 1,5-diazabicyclo [4.3.0] non-5-ene (DBN).

Figure 4 compares the efficiency and the peak asymmetry for the mono- and diphosphorylated nucleosides obtained with TEA, DABCO, and DBN in a mobile phase that contained

ammonium dihydrogen phosphate buffer under LC conditions (Figure 4a) and with the addition of 0.1 mole fraction carbon dioxide to the mobile phase with a mobile-phase velocity of 1 mL/min. When compared to the data where no base was added, DBN provides the best results with a 12% to 18% increase in efficiency in LC for monophosphate nucleotides and 111% to 320% for the diphosphates. In EFLC, the efficiency increased from 9% to 39% for monophosphates, and from 67% to 115% for diphosphates. For both HPLC and EFLC, tailing was the major contributor to low efficiency for the phosphorylated compounds; the impact of base addition on the asymmetry of the peak was studied to better understand the impact of each base on efficiency. DBN provided both the greatest decrease in peak asymmetry and increase in efficiency, followed by DABCO. Both bases greatly improved the peak shape while marginally affecting the retention of the analytes; no significant decrease in k was observed in EFLC, and an average 10% decrease in k was observed in LC. TEA either increased the peak asymmetry or did not impact it significantly.

Similar to other HILIC separations of polar compounds, the addition of a salt to the mobile phase was quite important. Sodium chloride (0.02 M) was used to assist the formation of the adsorbed water phase, and 75 mM ammonium phosphate was used to decrease the retention of the phosphate groups. Figure 5 shows that carbon dioxide expands the elution window for the compounds.

Summary

Now that SFC chromatographic instruments have a much larger range of operating pressures with accompanying precise temperature control, it is time to consider using liquefied gases such as carbon dioxide in mobile phases for a broader range of chromatographies. The lower viscosity of mobile phase and increased diffusivity for analyte separations using 0–100% liquefied gas provides decreased analysis time and often improved efficiency. The entire

range of this solvent continuum is valuable for separation science. A continuous change in viscosity, solvent strength, diffusivity, and permittivity occurs across this range of mobile-phase compositions. In particular, EFLC mobile phases are also providing value for the separation of highly polar compounds with similar operating parameters as in conventional HPLC. While advantages in performance are often noted when using acetonitrile–water for reversed-phase HPLC and HILIC compared to alcohol–water mixtures, the addition of carbon dioxide to the alcohol–water mixtures improves the chromatographic performance significantly. Also, both alcohol–carbon dioxide and alcohol–water–carbon dioxide are environmentally friendly.

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An Accurate-Mass Database for Screening Pesticide Residues in Fruits and Vegetables by Gas Chromatography–Time-of-Flight Mass Spectrometry

The main objective of this study was to evaluate the capabilities of gas chromatography (GC) with time-of-flight mass spectrometry (MS) for screening pesticides in fruits and vegetables using a purpose-built accurate-mass database. Analytical performance was tested on four matrices: potato, tomato, spring onion, and orange. Two resolution modes, 7000 FWHM and 12,000 FWHM, were tested to establish the concentration range within which automatic identification was possible, considering the retention time window of 0.2 min and at least two ions with a mass error lower than 10 ppm as the identification criteria. The effects of the matrix on identification and quantification were also studied for the four selected matrices. The developed method was applied to real samples and the qualitative and quantitative results were compared with those obtained using GC with triple-quadrupole MS.

Noelia Belmonte, Samanta Uclés, Miguel Gamón, Carmen Ferrer, Milagros Mezcua, and Amadeo R. Fernández-Alba

The use of agrochemicals at various stages of crop cultivation has an important impact on food protection and quality preservation. For this reason, there are different regulations in each country on pesticide use but the lists of approved pesticides are not harmonized worldwide. This situation is becoming more important because the number of pesticides authorized in Europe (1) has been reduced to around 50% of the total number of compounds manufactured. The practical “target analysis” approach applied in many routine laboratories for pesticide residue testing consists of selecting a list of compounds that are amenable for analysis by gas chromatography (GC) and liquid chromatography (LC) using an established combined priority list. This means that the majority of low-frequency or misused compounds are not studied.

GC coupled to mass spectrometry (MS) is the technique of choice for the analysis of many classes of pesticides, particularly volatile, semivolatile, and low-polarity compounds. The large number of GC–MS applications reported for pesticides analysis in fruits and vegetables (2–7) is the result of efficient GC separation, together with spectral information and satisfactory sensitivity provided by MS. Until recently, low-resolution single-quadrupole MS detectors working in selected-ion monitoring (SIM) mode were the instruments most commonly used because of their relatively low cost, compactness, and simplicity (8).

Single-quadrupole analyzers show limitations in analyzing complex matrices, however ion-trap detection and, more recently, triple-quadrupole analyzers allow operation in tandem MS (MS^2) mode, which achieves high sensitivity and selectivity, as demonstrated in an increasing number of applications reported in various fields (9,10). Recent progress in instrumentation design (mainly optics), the use of fast recording electronics, and signal processing improvements also have led to a renaissance for time-of-flight mass spectrometry (TOF-MS) in the study of organic compounds in complex matrices, particularly in LC-based applications. TOF-MS provides greater sensitivity in full-spectrum acquisition mode compared to conventional scanning instruments, principally because of its high mass-analyzer efficiency: GC–TOF-MS can screen hundreds of compounds at high sensitivity in a single run.

In addition, GC–TOF-MS data can be acquired and reprocessed without prior knowledge of the presence of certain compounds (that is, no analyte-specific information is required). Equally important is that the presence of other compounds of interest can be investigated at any time, simply by reprocessing the data (known as *retrospective analysis*). The high mass-resolving power and mass accuracy provided by GC–TOF-MS make it feasible to obtain extracted ion chromatograms using narrow mass windows—thus excluding a large proportion of the chemical background and isobaric interferences, which significantly improves signal-to-noise

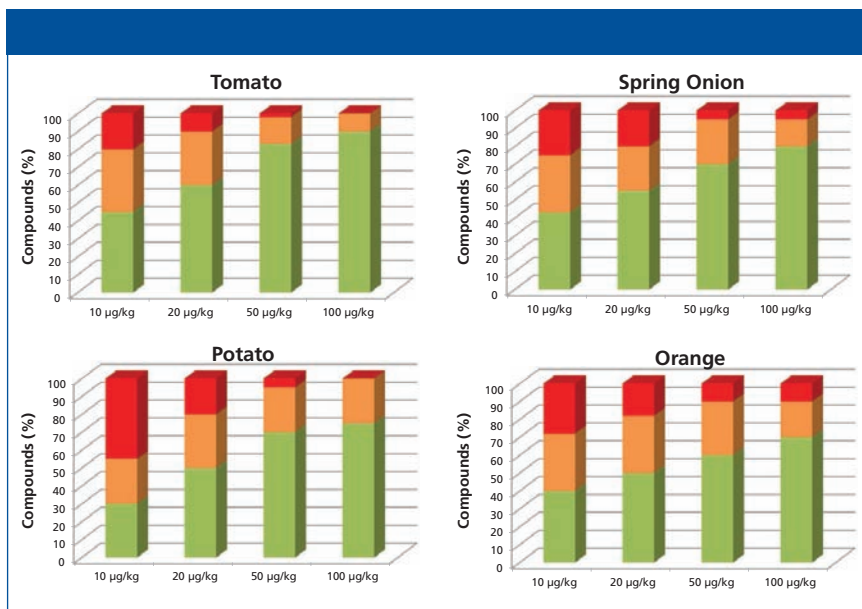


Figure 1: Percentage of compounds included in the databases identified automatically with more than two ions (green), one ion (orange), and not identified (red), working at low resolution.

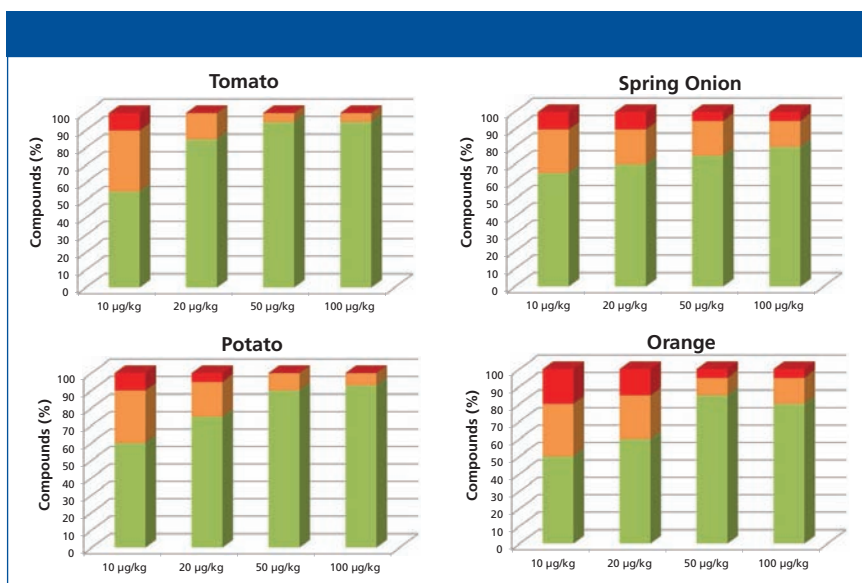


Figure 2: Percentage of compounds included in the databases identified automatically with more than two ions (green), one ion (orange), and not identified (red), working at high resolution.

ratios. Under these conditions, pesticide identification is improved in comparison to other conventional analyzers.

Over recent years, TOF-MS has been widely used for the development of screening methods for pesticide determination in fruits and vegetables, although LC-based methods are still more widely used (11,12). Nonetheless, compounds that exhibit a very low ionization yield with electrospray sources and that have low LC-MS amenability typically are outside the scope of those

LC-MS screening methods.

Nowadays, GC-TOF-MS is becoming a technique of interest within the scientific community for the determination of organic compounds (13,14). The main objective of this study was to develop a purpose-built database that includes the retention time and at least two ions for a total of 110 pesticides and to use this database in the identification of these compounds in fruit and vegetable matrices at different resolving powers.

Experimental

Chemicals and Reagents

All pesticide analytical standards used in this study were purchased from Dr. Ehrenstorfer and Sigma Aldrich at analytical grade (purity > 95%). A mixture standard solution contained all the pesticides studied and was prepared at 10 µg/mL in ethyl acetate and stored at -20 °C. Ethyl acetate was obtained from Fluka Analytical Pestanal.

Sample Treatment

The tomatoes, spring onions (called *scallions* in the United States), potatoes, and oranges used in this study were obtained from an organic farm in Almeria, Spain. The fruit and vegetable samples used for the application of the developed method were purchased from local markets.

The extraction method employed (15) is as follows: A representative 10-g portion of previously homogenized sample was weighed in a 200-mL PTFE centrifuge tube. Then 10 mL of ethyl acetate was added, and the tube was shaken vigorously for 3 s by hand. Next, 1.5 g of sodium chloride and 8 g of magnesium sulfate were added, and the tube was shaken in an automatic axial extractor (Agytax, Cirta Lab) for 15 min. The extract was then centrifuged at 3700 rpm for 5 min. Then the extract containing the equivalent of 1 g of sample per milliliter in 100% ethyl acetate was injected directly into the GC system.

Gas Chromatography

The separation of the pesticides from the whole fruit or vegetable extract was carried out using an Agilent 7890 GC system with two 15 m × 0.25 mm, 0.25-µm d_f Agilent HP-5MS UI Ultra Inert GC columns connected through an auxiliary programmable control module (PCM).

The samples were injected in splitless mode through an ultra-inert liner with a glass wool frit (Agilent). The injection volume was 2 µL. The injector temperature was held at 280 °C during the entire run time. Helium (99.999% purity) was used as the carrier gas. The oven temperature program was as follows: 60 °C for 1 min, then 60–120 °C at 40 °C/min, and 120–310 °C at 5 °C/min. The analytical separation

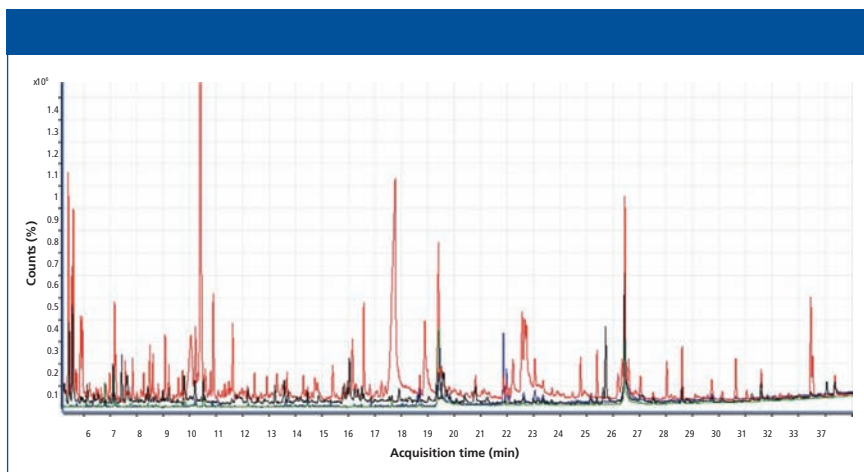


Figure 3: Total ion chromatogram for orange (red), tomato (blue), potato (green), and spring onion (black) obtained by GC–TOF–MS.

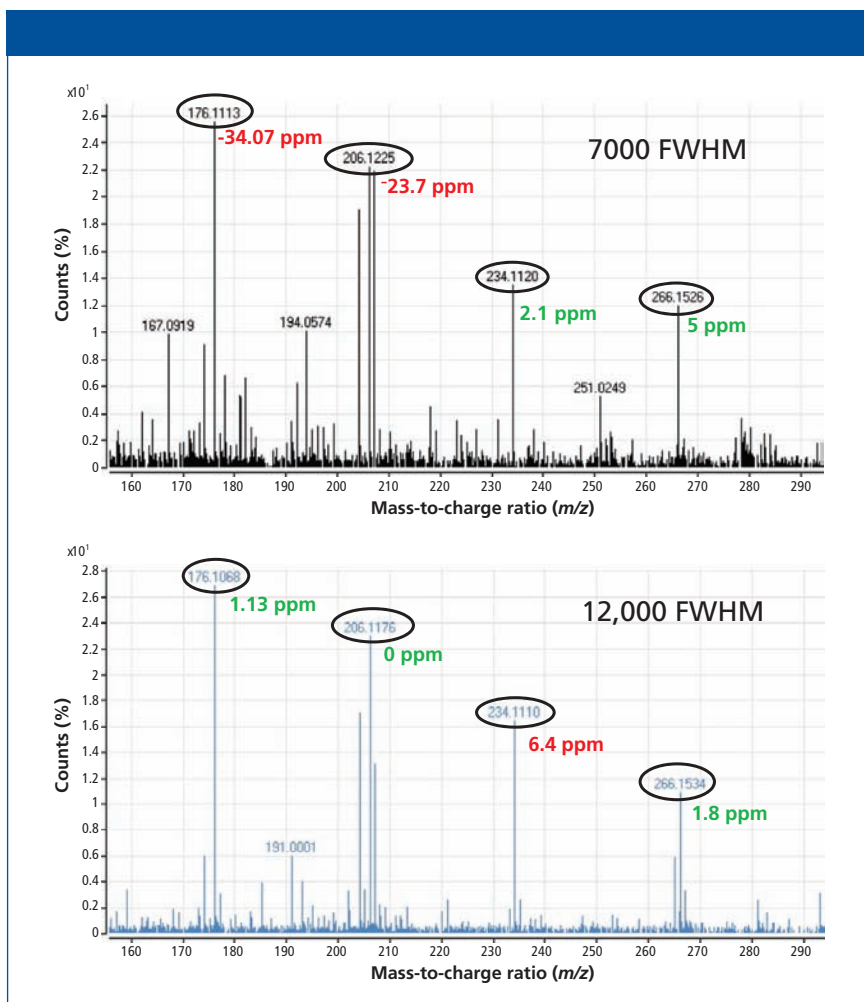


Figure 4: Mass spectra for benalaxyl in tomato at 20 µg/kg obtained at low and high resolution using GC–TOF–MS.

was performed under retention time locking conditions, using clorpyrifos methyl as the locking compound at a retention time of 18.11 min. The flow rates in col-

umn 1 and column 2 were 1.225 mL/min and 1.425 mL/min, respectively. The total run time was 40.5 min with an additional 3 min for backflushing.

Backflushing was carried out to eliminate unwanted heavy materials from column 1, thereby shortening the analysis time, reducing system maintenance, and prolonging column life. The backflush conditions were set as follows: the oven temperature was set at 310 °C, the PCM pressure was 50 psi, and the inlet pressure was 1 psi. These conditions allow a negative flow on column 1.

Mass Spectrometry

The GC system was connected to an Agilent Technologies model 7200 TOF–MS instrument equipped with an electron ionization source. The ion source and quadrupole analyzer temperatures were set at 280 °C and 150 °C, respectively. The TOF analyzer was operated at two different acquisition rates, 2 GHz and 4 GHz, acquiring data in the m/z 45–550 mass range. Perfluorotributylamine was used for daily MS calibration. The accuracy of the generated ions was controlled through an internal mass calibration performed before each injection.

Creation of the Database

The experimental conditions described above were applied to create an accurate-mass database of 110 pesticides. The selected pesticides were run in a tomato matrix at a concentration of 100 µg/kg. The full-scan spectrum was studied and at least two characteristic ions (with a relative abundance higher than 30% with respect to the base peak) were selected. In most cases, the molecular ion of the pesticides was not present in the mass spectrum or its relative abundance was too low to be considered. After the ions were selected, a molecular formula was assigned, and their exact masses were calculated and used to create the database together with their retention times. All the information was collected in an Excel file, which was converted into a CSV format to be used as the library and linked to Mass Hunter data analysis software (Agilent Technologies).

Automatic Identification Using the Pesticide Database

The selected matrices were spiked with

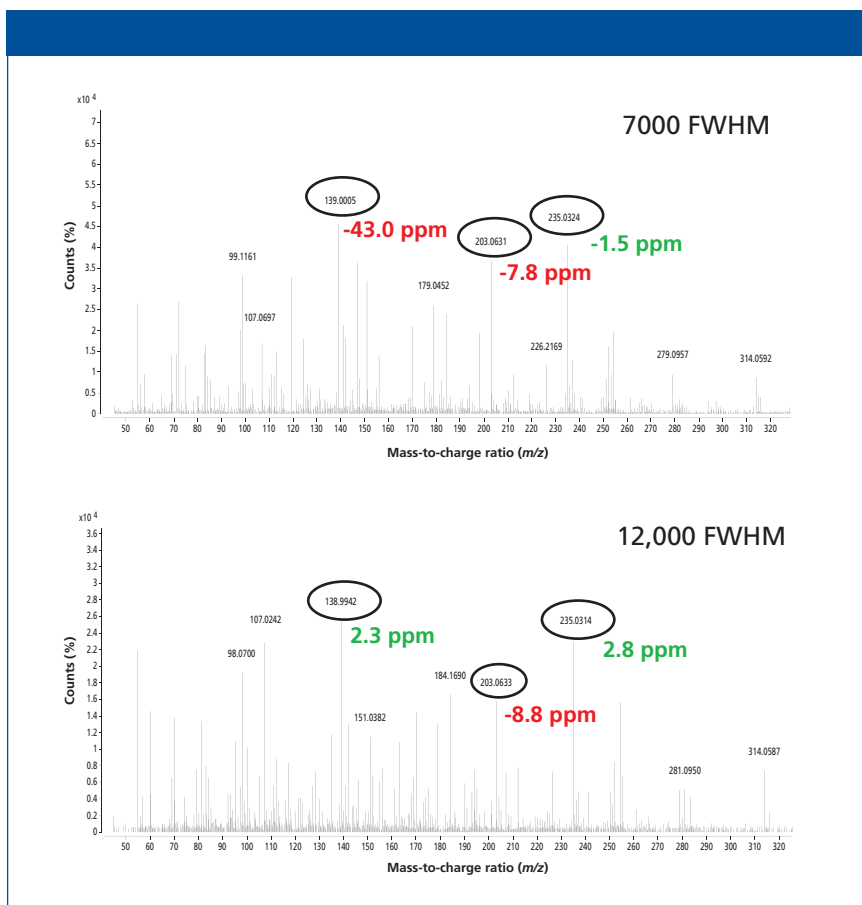


Figure 5: Mass spectra for nuarimol in orange at 20 µg/kg obtained at low and high resolution using GC-TOF-MS.

all the pesticides at four concentration levels, 10, 20, 50, and 100 µg/kg. Automatic identification was performed at two acquisition rates, 2 GHz and 4 GHz, which allowed respective mass resolutions of 7000 and 12,000 full width at half maximum (FWHM). The search parameters were 0.2 min for the retention time window and 10 ppm for the mass error tolerance.

Results and Discussion

The information obtained from this study has been discussed considering two different goals. The first was to perform screening intended to produce a yes-or-no answer while considering only two identification criteria: the retention time and a single ion with a mass accuracy of less than 10 ppm mass error. The second goal was to obtain confirmation of results and semiquantification considering two ions with a mass accuracy of less

than 10 ppm mass error and the correct retention time.

Goal 1: Screening and Automatic Identification

The four selected extracts spiked at 10, 20, 50, and 100 µg/kg were analyzed at low resolution (7000 FWHM) and at high resolution (12,000 FWHM). The analysis was processed with data analysis software using the purpose-built database as the library.

The pesticides, identified automatically with the correct retention time and one ion with a mass accuracy of less than 10 ppm mass error, were counted and compared with the total number of pesticides included in the database to calculate the percentage of pesticides identified.

The results obtained operating at 7000 FWHM are shown in Figure 1. The green columns for each concentration level are the pesticide percentages with at least two ions having an error

lower than 10 ppm and a retention time difference of less than 0.2 min. The orange columns are the pesticide percentages with at least one ion with a mass error lower than 10 ppm and a retention time error of less than 0.2 min. The columns in red are the compound percentages not detected or detected with a mass error higher than 10 ppm.

At the 10-µg/kg level, the percentages of pesticides identified with at least one fragment and the correct retention time were as follows: 80% in tomato, 50% in potato, 76% in spring onion, and 70% in orange. As expected, at higher concentration levels, the percentage of pesticides found was higher.

Figure 2 shows the results of the studied matrix at the different concentration levels obtained when operating the system at high resolution (12,000 FWHM). At this acquisition rate, the percentage of positives at all concentration levels, and in all matrices, is higher than when operating at low resolution. At 10 µg/kg, the percentages of pesticides identified with at least one fragment and the correct retention time were as follows: 88% in tomato, 91% in potato, 88% in spring onion, and 82% in orange. At 20 µg/kg, 100% of the pesticides included in the database for tomato were identified with at least two ions and a mass error < 10 ppm along with a retention time < 0.2 min. The pesticide identification rates for the spring onion, potato, and orange were 95%, 97%, and 85%, respectively. Operating at high resolution, the number of pesticides found with a mass error below the level established as the mass tolerance was higher than at low resolution.

An important influence on mass assignments is the ability of a mass spectrometer to resolve two peaks on the *m/z* scale when they are close together.

When peaks are not (fully) resolved, the resulting measured mass profile will be the sum of the two individual mass profiles, and the maximum of the combined profile will lie somewhere between the exact masses of the two individual peaks. As a consequence, the mass assignment, which is based on a centroiding algorithm

Table I: Real samples identified and quantified with the proposed method and compared to GC–triple-quadrupole MS. Values listed in parentheses are the concentrations that were found in the samples.

Sample	GC–TOF–MS	GC–Triple–Quadrupole MS	Comments
Pepper	Boscalid (0.25 mg/kg)	Boscalid (0.23 mg/kg)	
Tomato 1	Iprodione (0.20 mg/kg)	Iprodione (0.27 mg/kg)	
	Pyriproxifen (0.054 mg/kg)	Pyriproxifen (0.062 mg/kg)	
Tomato 2	Bifenthrin 1F (0.12 mg/kg)	Bifenthrin (0.13 mg/kg)	Ion 166.099 error > 10 ppm
Melon 1	Bupirimate (0.15 mg/kg)	Bupirimate (0.13 mg/kg)	
Tomato 3	Buprofezin (0.49 mg/kg)	Buprofezin (0.16 mg/kg)	
	Metalaxyl (0.060 mg/kg)	Metalaxyl (0.068 mg/kg)	
Zucchini	Pirimicarb (0.33 mg/kg)	Pirimicarb (0.24 mg/kg)	
	Bupirimate (0.11 mg/kg)	Bupirimate (0.048 mg/kg)	
	Penconazole (0.091 mg/kg)	Penconazole (0.074 mg/kg)	
Brussels sprouts	Boscalid (0.21 mg/kg)	Boscalid (0.12 mg/kg)	
Tomato 4	Bifenthrin (0.10 mg/kg)	Bifenthrin (0.125 mg/kg)	Ion 166.099 error > 10 ppm
Bean 1*	Iprodione 1F (0.830 mg/kg)	Iprodione (1.02 mg/kg)	After dilution
Bean 2	Azoxystrobin ND	Azoxystrobin (0.337 mg/kg)	Coelution
	Iprodione (0.36 mg/kg)	Iprodione (0.25 mg/kg)	
Bean 3	Azoxystrobin ND	Azoxystrobin (0.11 mg/kg)	Coelution
	Iprodione (0.19 mg/kg)	Iprodione (0.078 mg/kg)	
Melon 2	Azoxystrobin ND	Azoxystrobin (0.16 mg/kg)	Coelution
	Bupirimate (0.11 mg/kg)	Bupirimate (0.08 mg/kg)	
Bean 4*	Iprodione (1.82 mg/kg)	Iprodione (1.70 mg/kg)	After dilution
Bean 5*	Iprodione (1.27 mg/kg)	Iprodione (1.70 mg/kg)	After dilution
Grape	Iprodione (0.15 mg/kg)	Iprodione (0.11 mg/kg)	
	Myclobutanil (0.041 mg/kg)	Myclobutanil (0.041 mg/kg)	

*These samples were injected after being diluted 1:2 to achieve correct identification and quantification.
1F = one fragment detected; ND = not detected

of the detected profile, will result in an incorrect analyte mass. As sample complexity becomes greater (a larger number of matrix peaks at high levels relative to the analytes), the mass resolution (defined as an MS instrument's ability to distinguish between two ions with similar m/z values) of a mass spectrometer can become a key parameter in the correct assignment of analyte masses.

Figure 3 shows the full-scan chromatogram of the four matrices selected for this study. The total ion chromatogram in red corresponds to the orange matrix; the chromatogram in black corresponds to spring onion; green corresponds to potato; and blue corresponds

to tomato. Orange shows the largest number of matrix interferences.

Figure 4 is an example showing the analysis of benalxyl at 20 ppb in tomato analyzed at both low and high resolution. If we set the mass tolerance to 10 ppm, only two ions are identified when operating at 7000 FWHM, whereas all the ions are identified at 12,000 FWHM.

Figure 5 is an example of an analysis in the orange matrix. In this case the mass spectrum of the pesticide nuarimol is shown at low and high resolution. At low resolution only one ion is correctly identified, whereas at high resolution two ions were identified with a mass error lower than that established.

Goal 2: Confirmation and Semiquantification

With the purpose of using the developed method for confirmation and semiquantification, we considered the identification criteria to be the identification of at least two ions with a mass error lower than 10 ppm and a retention time error lower than 0.2 min. The pesticide percentages that meet these criteria are as follows: Operating at high resolution, more than 70% of the pesticides were detected at the 20 µg/kg level in tomato, potato, and spring onion (Figure 2) with a mass error lower than 10 ppm, and with a retention time error lower than 0.2. In the orange, 60% of the pesticides were detected.

After it was known that identification and further confirmation worked far better at high resolution, the linearity of the response was studied between 10 and 100 µg/kg operating at high resolution, and very good linearity was observed in this range, showing correlation coefficients better than 0.999, except in these specific cases: benalaxyl, tetraconazole, and flutolanil in orange; butralin, etoxyquine, and *p,p'*-DDT in tomato; and pirimifos methyl, pyriproxyfen, tetraconazole, tetradifon, and tolylfluanide in spring onion.

Given that screening methods are intended to be used with a large number of matrices, a semiquantification study of samples was performed in the tomato matrix-matched solution at two concentration levels, 10 and 100 µg/kg. The results were compared with those obtained using a GC–triple-quadrupole MS system in which the concentration of the samples was calculated with a calibration curve in matrix-matched solution performed with different commodities, depending on the analyzed samples.

The selection of tomato as the quantification matrix was based on the results obtained from a matrix effect study. The slope of all the matrices obtained from the linear curve in the range between 10 and 100 µg/kg was compared with the slope obtained in solvent. All matrices showed a marked signal enhancement effect when compared with solvent; however, the differences in the slopes between matrices were very small.

Real samples were analyzed with the developed method, and some of the pesticides found are listed in Table I. The results were compared with those obtained by GC–triple-quadrupole MS; the samples were also semiquantified as described above, and the results obtained are also shown in Table I. The quantification differences for both systems are mostly within 50%. In some cases the pesticide concentration was high, which caused detector saturation; such samples were injected after dilution for better identification and

quantification. This was the case of iprodione in the bean samples. Another special case was bifenthrin in tomato; this compound was identified with only one ion because other characteristic ions showed errors higher than those established.

Conclusions

The created database of 110 pesticides, which included the retention time and at least two ions, was applied to automatic pesticide identification in tomato, potato, spring onion, and orange. This automatic identification was made and compared at two resolution powers, showing better results in high-resolution mode. The identification of nearly 100% of pesticides included in the database was demonstrated at low concentration levels (10 and 20 µg/kg) in all the studied matrices except in orange, where the pesticide identification percentage was 85%. In addition, the method was applied for the analysis of real samples, and the obtained results were compared with those using GC–triple-quadrupole MS, showing differences in the quantification results of less than 50%.

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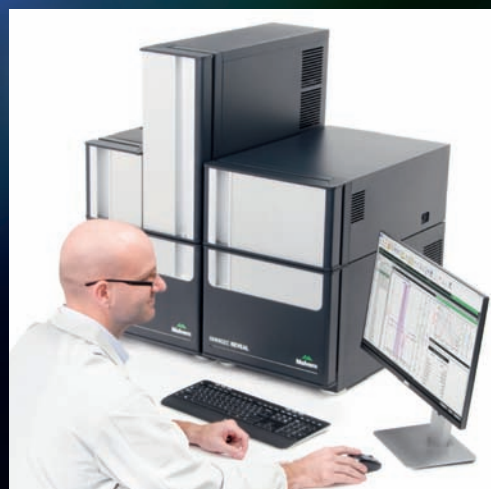
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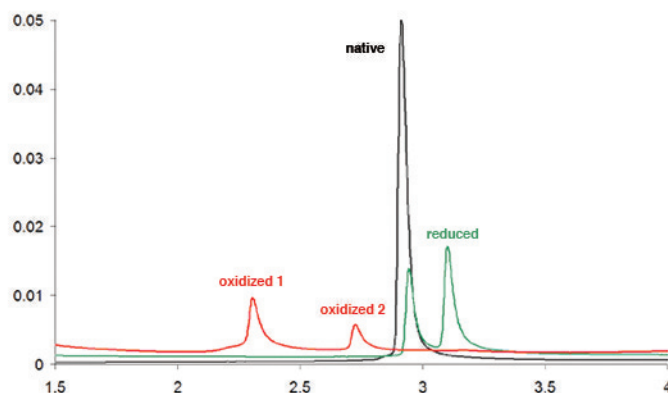


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